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The Organization Of The Capillary Network In Skeletal Muscle

Michael John Plyley

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THE ORGANIZATION OF THE CAPILLARY NETWORK IN SKELETAL MUSCLE

by

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Department of Biophysics

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
April 1977

In memory of my Father

ABSTRACT

Oxygen transport to the fibres of striated muscle is dependent on blood flow in capillaries and oxygen diffusion in tissue. To understand the process fully requires a detailed knowledge of the three-dimensional arrangement of the capillaries, and their precise relationship to the fibres. The capillaries in striated muscle run mainly parallel to the fibres and, in their course from arteriole to venule, branch and anastomose frequently. The axial symmetry of the tissue makes it possible to analyse capillary geometry by sectioning either parallel (longitudinal plane) or perpendicular (transverse plane) to the fibre axes.

The number of capillaries supplying individual muscle fibres of dog, cat, rat, rabbit, guinea pig, quail and frog was studied in transverse sections of striated muscle from the hindlimb or head. Each muscle was perfused under high pressure with Microfil (a silicone elastomer of viscosity 20 cP approx.) to outline the vascular bed. The muscles were fixed in formalin, embedded in paraffin and the 15 µm sections were stained by a modified Gomori trichrome stain. Examination of these sections showed most capillaries filled with Microfil as well as a few, remaining unperfused, containing red cells. From well-perfused bundles, the numbers of capillaries supplying each fibre were

counted; in all muscles, these ranged from zero to nine and were normally distributed. The mean values for each muscle ranged from 2.9 to 4.0, and the grand mean of the frequency distribution was 3.47 ± 1.11 (SD; n = 100). The number of fibres "sharing" one capillary ranged from two to six; the mean values for each muscle varied between 2.0 and 3.2, the lower value being found when four vessels surrounded a fibre and the higher value when there were only three. The overall mean "sharing" factor was 2.48 ± .31 (SD; n = 19).

The results indicate that the relationship of capillaries to fibres in muscle is described by a continuum of transitional models from a square array of fibres with each capillary located between two adjacent fibres to a hexagonal array with capillaries at alternate vertices. This was true of both 'red' and 'white' muscle.

Analysis of the capillary branching pattern and the dimensions of the capillary network in the longitudinal plane were studied in sartorius muscle of frog. The many anastomoses in the network provide numerous distinct routes by which blood may pass from arteriole to venule. Measurement of each segment in the network, made it possible to determine the total length of each of these routes, by simple summation of the lengths of the segments involved. The distribution of these path lengths was Gaussian, the mean value being 3.58 ± 1.64 mm (SD; n = 238). The number of segments comprising a pathway ranged from one to nine, the mean value was 4.44 ± 1.84 (SD). The number of branching points per path (one less than the number of segments) followed a Poisson distribution and, therefore, the distribution of segment lengths was exponential. Mean segment length was 0.85 ± .64 mm

(SD; n = 620). This suggests that during growth, the branching of the capillary network occurs on a random basis along the length of the capillary.

Measurements were made, at each successive branching point, of the number of capillaries connected, the frequency of anastomosis between capillaries deriving from the same or different parent arterioles, the frequency of convergent and divergent branchings and the angles of branching. The mean angles of branching were 58.9 ± 24.9° (SD; n = 81) for convergent branches and 60.1 ± 28.3° (SD; n = 85) for divergent branches. In contrast to the branching of a simple binary system, the capillary network diverges immediately at the terminal arteriole and passes, via numerous interconnecting parallel pathways, to the venule. This leads to a more compact network without areas of insufficient supply.

ACKNOWLEDGEMENTS

A thesis is not the end product of one individual's efforts but results from the efforts of a number of people working towards a common goal. As one nears the end of an endeavour such as this, it is only natural to reflect upon the last four or five years and the people who make this thesis a reality.

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"In order to solve this general problem or the more modest part of it..., the first thing to do must be to find out about the number, distribution and surface of capillaries in those tissues in which we are interested.

For information of this kind we naturally turn to the anatomical literature, but I regret to say that in the main we are disappointed. We cannot find there that quantitative information which we require."

August Krogh (1922)

"It would appear that almost half a century after Krogh's plea for 'quantitative anatomy', physiologists must obtain the facts about capillary number, distribution and arrangement by direct observation if further progress is to be made."

Martini and Honig (1969)

INTRODUCTION

With few exceptions, the fundamental unit of living material is the cell. Specialized structures within each cell (organelles) endow the cell with the capacity to carry on basic life processes and to reproduce itself. For each cell, the supply of basic raw materials and the removal of the waste products of metabolism are dependent on the process of diffusion.

Diffusion is that spontaneous movement of matter that brings about uniformity of concentration everywhere within the system. Stated simply, diffusion is the movement of material from an area of high concentration to another area of lower concentration in accordance with the second law of thermodynamics. Thus the direction of movement of material at any point within the system will be in the opposite direction to that of the gradient of concentration. The rate of movement of material will be proportional to both the magnitude of the concentration gradient and the size of the area (perpendicular to the direction of movement) through which diffusion is taking place. The constant of proportionality is termed the diffusion constant. Mathematically, the relationship is expressed by Fick's equation:

$$\frac{\partial m}{\partial t} = -DA\left[\frac{\partial c}{\partial x}\hat{i} + \frac{\partial c}{\partial y}\hat{j} + \frac{\partial c}{\partial z}\hat{k}\right] \qquad (1)$$

where $\frac{\partial m}{\partial t}$ is the rate of movement of the diffusing material (in moles/min), D is the diffusion constant (in cm^2/min), A is the area of surface across which diffusion is occurring (in cm^2), $\frac{\partial c}{\partial x}$, $\frac{\partial c}{\partial y}$, $\frac{\partial c}{\partial z}$ are the x, y and z components of the concentration gradient (in moles/ml/cm) and $\hat{i}$, $\hat{j}$, $\hat{k}$ are the unit vectors parallel to the x, y and z axes. The negative sign indicates the motion of the material is down the gradient of concentration.

Thus far it has been assumed that the diffusing material is neither produced nor consumed by the cell. In such cases, the concentration gradient will be dependent on the rate of production or consumption within the cell (see Ackerman, 1962, pp. 419-429).

When discussing diffusion of a gas, it is better to speak of differences in the partial pressure of the gas rather than differences in concentration. This is because of wide variations in solubility within the tissue. The concentration of the gas is proportional to its partial pressure; the constant of proportionality (α) is the solubility coefficient (in moles/ml/atmosphere (STP)). The relationship is given by the expression $C_{O_2} = \alpha P_{O_2}$

The dependence of cells on the process of diffusion places an upper limit on the maximum size of cells. Provided that a cell exhibits isogonic growth, its requirements for oxygen and other nutrients will be proportional to the volume of the cell, ie. to the $(\text{length})^3$; however, the supply of these materials by diffusion is dependent on the surface area of the cell through which the substance must diffuse, and this varies as the $(\text{length})^2$. Thus, as the cell increases in size, its demands for nutrients would eventually exceed the supply, resulting

in regions of deprivation within the interior of the cell. It is possible, using the one-dimensional form of the Fick equation for a respiring tissue (Jacobs, p.138), to calculate the maximum distance that diffusion can transport oxygen in a tissue in face of the rate of oxygen consumption throughout the tissue. This calculation yields the result that this maximum diffusion distance is 0.58 mm.

Clearly, if cells are to increase in size, the problem of supply and demand must be overcome. Examination of the variables which influence the diffusion of a gas reveals that several parameters are constrained by the physical world. Both D and α are temperature-dependent, but within a given tissue, they may be regarded as constant. Furthermore, as the atmospheric partial pressure of oxygen varies little at any given altitude, the normal arterial partial pressure of oxygen (P_{a0_2}) in the blood may be regarded as constant. Finally, it is most unlikely that tissue metabolism can be decreased by any appreciable amount. Therefore, to increase the rate of diffusion, it is necessary to increase the surface area (A) ghrough which diffusion takes place, or increase the partial pressure gradient $\frac{dP0_2}{dx}$ by decreasing the distance over which diffusion takes place (dx).

Nature's solution to the problem was the evolution of multi-cellular organisms in which groups of cells have become specialized to perform particular functions within the organism. To provide adequate transport of raw materials to and from each cell, a circulatory system was developed. This system is composed of two parallel circuits of vessels, the pulmonary and systemic circulations, through which blood is propelled by the heart (Figure 1). Each circuit is composed of a series

of distributing and collecting vessels, arteries and veins, joined by a network of exchange vessels, capillaries (Figure 2). The network of exchange vessels consists of a multitude of minute vessels connected in parallel rather than series. Although the surface area available for diffusion from one such vessel is small, having many such vessels connected in parallel leads to a vast area available for diffusion. This arrangement also ensures that the diffusion distance is maintained as small as possible. Thus Nature has designed an exchange system which has maximized the product $(A \cdot \frac{dP_{O_2}}{dx})$ within each tissue in order to ensure adequate transportation of materials.

Exchange of materials between capillaries and cells is achieved, as in unicellular organisms, solely by the process of diffusion. However, in the case of tissues, diffusion occurs from a long, narrow cylinder (capillary) and proceeds radially outward into a large cylinder of tissue. As the rate of bulk flow of blood is very much greater than the rate of diffusion, it can be assumed that diffusion down the length of the vessel is negligible. Solving the Fick equation for this case of cylindrical symmetry yields the result:

$$P_{O_2}(\text{cap}) - P_{O_2}(x) = \frac{q'}{D\alpha}\left[\frac{R^2}{2} \ln \frac{x}{r_c} - \frac{x^2 - r_c^2}{4}\right] \qquad (2)$$

where $P_{O_2}(\text{cap})$ and $P_{O_2}(x)$ are the partial pressures of oxygen in the capillary and at a point x in the tissue (in atmospheres), R is the diffusion distance (equal to one-half the distance between adjacent capillaries) and r_c is the radius of the capillary. In the limiting case, when $P_{O_2}(x)$ equals zero and $x = R$, the equation becomes:

Figure 1 - Schematic representation of the mammalian circulatory system illustrating the two circuits of which the circulation is composed.

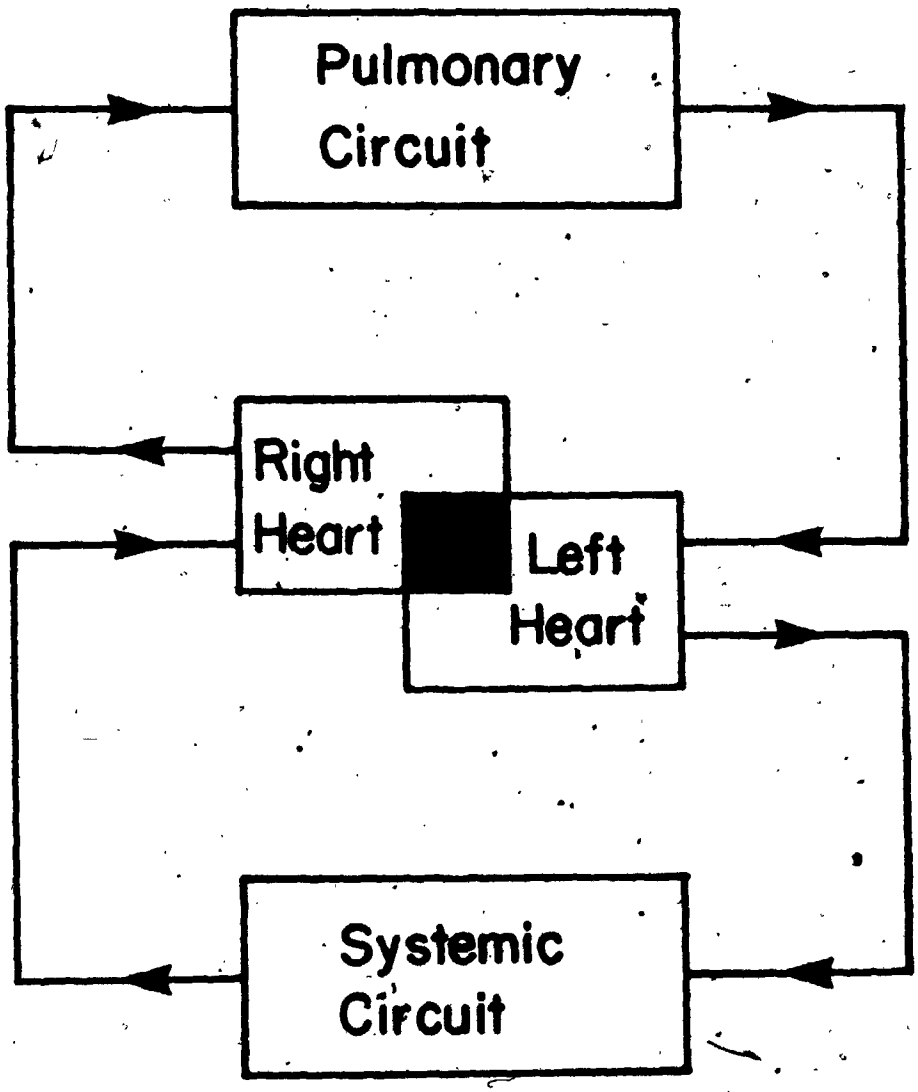

Pulmonary
Circuit
Right
Heart
Left
Heart
Systemic
Circuit

Figure 2 - The primary vascular components of each circuit are the arterial, capillary and venous vessels, which serve the functions of distribution, exchange and collection. While the distributing and collecting vessels are connected in series, the exchange vessels are joined together in parallel, forming a network of inter-connected pathways linking the arterial and venous systems.


1 mm

$$P_{O_2}(\text{cap}) = \frac{q'}{D\alpha}\left[\frac{R^2}{2}\ln\frac{R}{r_c} - \frac{R^2 - r_c^2}{4}\right] \qquad (3)$$

This equation is commonly known as the Krogh-Erlang equation, and has been the basis of most models of diffusion of oxygen from capillaries into tissue for a number of years (Figure 3).

Skeletal muscle comprises 40-50% of total body mass. As this tissue requires a large amount of oxygen, particularly during activity, its dependence on an efficient gas transport system is substantial. Therefore, an analysis of the arrangement of the exchange vessels in skeletal muscle could contribute to a better understanding of the principles underlying oxygen transport to this tissue.

Figure 3 - Schematic representation of the relationship between the variables influencing tissue oxygenation. Radial diffusion of oxygen from a capillary to any point (x) in a tissue is dependent upon several factors:

(i) the difference in the partial pressure of oxygen between the capillary ($P_{O_2}(cap)$) and that at point x ($P_{O_2}(x)$).

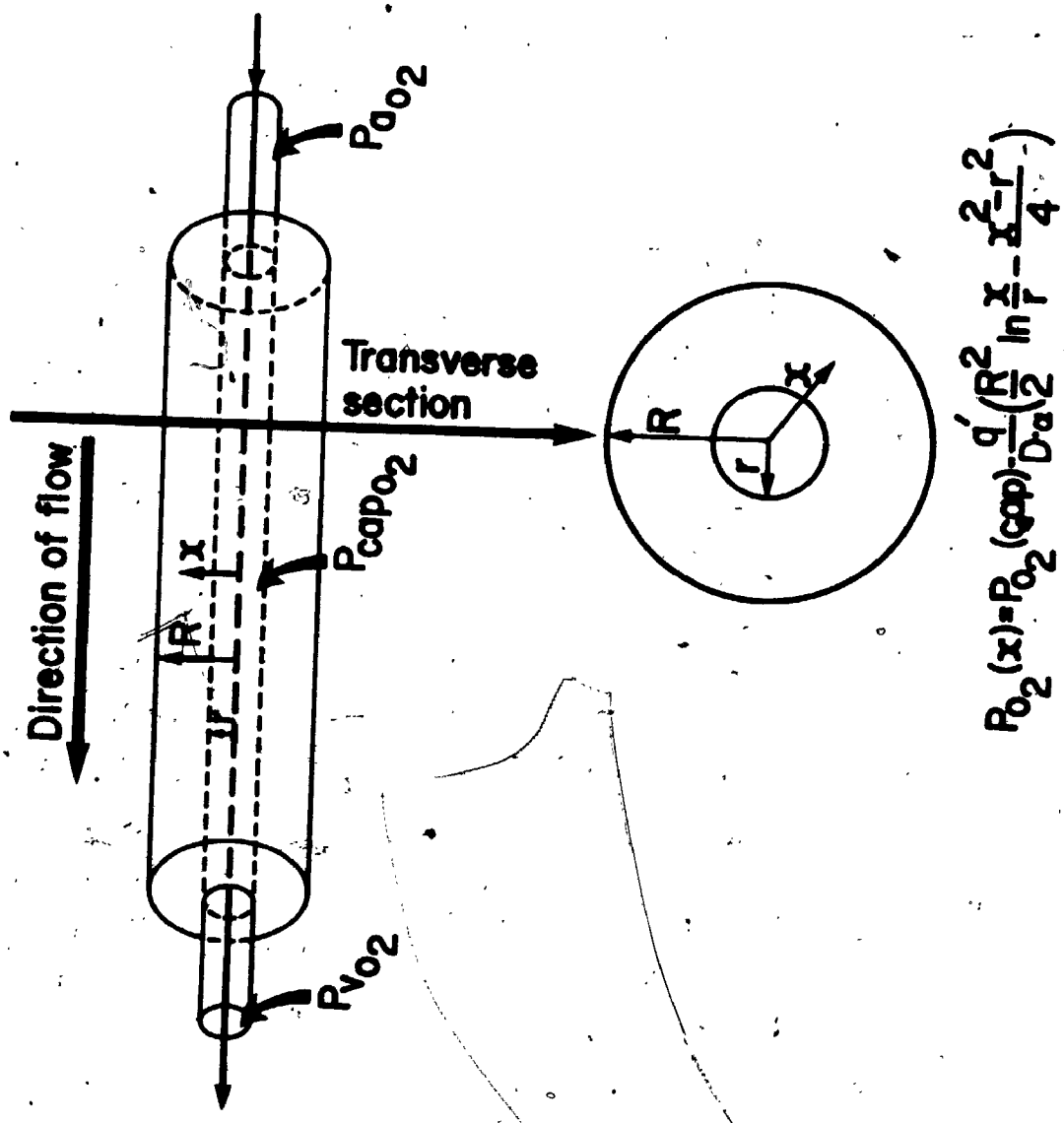
(ii) the oxygen consumption of the tissue (q').

(iii) the diffusion constant (D) and solubility (α) of oxygen in the tissue.

(iv) the distance between capillaries which are being perfused at any instant (2R) and

(v) the radius of the capillary (r).

These variables are related by the well-known Krogh-Erlang equation.


P_{O_2}
Transverse section
Direction of flow
$P_{cap_{O_2}}$
x
R
L
$P_{V_{O_2}}$
R
x
r
$P_{O_2}(x) = P_{O_2}(cap) - \frac{q'}{D_a}\left(\frac{R^2}{2}\ln\frac{x}{r} - \frac{x^2-r^2}{4}\right)$

"I often say that when you can measure what you are speaking about and express it in numbers you know something about it; but when you cannot express it in numbers your knowledge is of a meagre and unsatisfying kind: it may be the beginning of knowledge, but you have scarcely, in your thoughts, advanced to the state of science."

Lord Kelvin (1883)

(quoted by Aherne, 1970).

I. HISTORICAL REVIEW

I.1 The blood capillary

It is generally believed that Harvey's treatise on the movement of blood from the heart, through the arteries, into the veins and back to the heart represents the first account of a blood circulation. Such is not the case, for several notable scientists of the past, although they could not prove its reality, reasoned that blood must pass from artery to vein (or vein to artery) by some means. As stated by Victor Robinson (1929):

> "We are not in the habit of thinking that the capillary connection between the arteries and the veins was known in antiquity, but let us not be startled at anything we find among the Greeks. In De Usu Partuim Galen wrote: 'The arteries and veins anastomose with each other throughout the body, and exchange with each other blood and spirits by certain invisible and exceedingly small passages'."

Thus, if this translation is correct, the concept of a capillary connection between arteries and veins existed fifteen hundred years before Harvey.

In the tenth century, a Muslim physician, Haly Abbas, suggested the existence of the blood capillary system, when he stated that there were "pores between the pulsating and non-pulsating vessels" (Castiglioni, 1947). His work, however, was lost to later scientists as it was not translated until this century; a pity, for it may have led to the momentous discovery of the capillaries five hundred years earlier.

Until the sixteenth century, many of Galen's concepts were rigidly adhered to, for few scientists challenged the great master. The feeling of the times was expressed by Vesalius in 1543: "Not long ago I would not have dared to diverge a hair's breadth from Galen's opinion" (Mason, 1953). However, Vesalius went on to point out that the septum is "as thick, dense, and compact, as the rest of the heart", and questioned Galen's idea that the blood passed from the right side of the heart to the left via pores in the septum. He did not, however, arrive at an alternate hypothesis. The correct explanation for the lesser circulation was provided by Servetus in 1550:

> "The communication between the ventricles, however, is not made through the midwall of the heart, but in a wonderful way the fluid is conducted by a long detour from the right ventricle, through the lungs... passes from the arteria venosa into the vena arteriosa, whence it is finally drawn by the diastole into the left ventricle" (Robinson, 1929).

Later Servetus postulated that there were not two forms of blood (differentiated by the natural and vital spirits) but "only one blood...which is communicated by the joins from arteries to veins" (Mason, 1953).

The greatest obstacle in the development of a theory for the circulation of blood was that such a theory required mixing of the two traditional fluids, which, until this time, had been thought to be separate entities, performing two distinct functions. Once it was realized that venous and arterial blood were one and the same, a theory for the circular movement of blood was inevitable. However, the theory was not postulated until 1628.

Harvey had felt for some time that the movement of blood must be in a circle, as many naturally occurring phenomena had been found to be cyclic.

> "I began to think whether there might be a motion as it were in a circle in the same way as Aristotle says that the air and the rain emulate the circular motion of the superior bodies... and in like manner are tempests and meteors engendered by the circular motion, and by the approach and recession of the sun. And so in all likelihood does it come to pass in the body, through the motion of the blood." (Mason, 1953).

Harvey, through simple calculations, concluded that the amount of blood which the heart pumped was much greater than could be accounted for from the continuous breakdown of foodstuffs (ingesta). He also observed that the arteries, not the veins, dilated with each beat of the heart and concluded that the flow must be from the heart, down the arteries, somehow into the veins, and back to the heart. In his book, On the Motion of the Heart and Blood, Harvey wrote:

> "I must refer to certain experiments which make it clear that the blood goes into each member through the arteries and flows out of it through the veins; that the arteries are the vessels which carry blood away from the heart, and the veins the vessels and pathways for the return of the blood to the same heart; that in the members and extremities the blood passes from the arteries into the veins either directly by anastomosis, or indirectly through porosities of the flesh, or in both ways, just as it passes from the veins into the arteries in its cardio-pulmonary course. Hence it is manifest that it moves from one region to a second and back again, that is to say, from the centre to the farthest parts and thence back to the centre." (Franklin, 1957)

Yet Harvey, like others before him, was unable to provide details concerning the mysterious connection between the arteries and the veins.

The "missing link" in the theory was discovered in 1660 by Malpighi, and independently in 1683 by van Leeuwenhoek, and their course from artery to vein was traced in specimens injected with coloured fluids or liquid wax by Boyle in 1663. The excitement of the discovery can be felt when reading the words of Malpighi:

> "I saw the blood, showered down in a tiny stream through the arteries, after the fashion of a flood, and I might have believed that the blood itself escaped into an empty space and

> was gathered up by a gaping vessel, but an objection to this view was offered by the movement of the blood being tortuous and scattered in different directions and by its being united again in a determinate part. My doubt was changed to a certainty by the dried lung of a frog which to a marked extent had preserved the redness of the blood in very minute tracts, which were afterwards found to be vessels, where by the help of a glass I saw not scattered points but vessels joined together in a ring-like fashion. And such is the wandering about of these vessels as they proceed on this side from the vein and on the other from the artery that the vessels no longer maintain a straight direction, but there appears a network made up of continuations of the two vessels.....Hence it was clear to the senses that the blood flowed along sinuous vessels and was not poured into spaces, but was always contained within tubules, and that its dispersion is due to the multiple winding of the vessels." (Robinson, 1929)

This last discovery not only provided proof for the theory that blood circulates, but also completed studies on the mechanics of the circulation. Scientists now began investigating the significance of, and the role played by these "exceedingly minute passages". Van Leeuwenhoek speculated correctly that their function was one of great importance to supplying the essentials needed by the body.

> "since the large arteries are thick-walled, they obviously cannot be sites of delivery of nourishment to the tissues. Rather must 'the particles of blood from which its redness

> proceeds' deliver their 'more subtile juices' through the capillary walls, 'whereby the blood, when returning in the veins, being deprived of those juices which are taken away, it will appear blackish'". (Graubard, 1964)

I.2 The capillary blood supply to skeletal muscle

The first observation of the movement of erythrocytes in muscle were made by Hales (1733) using the rectus abdominis of frog. Boerhaave (1774) reported that the blood content of some muscles (gross appearance - red) was greater than other paler muscles.

Crude blood flow measurements made by Fontana (1785) indicated that the blood flow in exercising muscle is greater than that in resting muscle. Gaskell (1877) measured the blood flow at rest and during exercise more precisely and concluded that the flow during exercise was indeed greater than that during rest. However, the mechanism(s) responsible for such changes in flow have yet to be elucidated.

The first studies on the architecture of the vascular bed in muscle were made by Ranvier (1873, 1874a, b, c, 1880) and Meyer (1875). Ranvier (1874b) noted that the vascular mesh (mailles rectangulaires) was different in red and pale muscle (translated by author):

> "The pale muscles show in the distribution of their blood vessels an arrangement represented by the following histological traits....The long branches of the capillary mesh run along the bundle, whereas the transverse branches form links between the segments. The red muscles possess

> blood vessels whose form and distribution differ from this classical description. The meshes are, on many points, nearly as wide as they are long. The long branches are tortuous and more variable than in the other muscle preparation. The transverse branches possess fusiform dilatations....One observes similar dilatations at the junction of several capillaries and in little veins. Sometimes the capillaries and venules are covered with these dilatations."

Meyer (1875) reported that the capillary network in red muscle was more extensive than that in pale muscle.

The classical description of the vascular architecture in muscle was given by Spalteholz (1888) (translated by author):

> "The arteries supplying a muscle branch freely, and between the branches there are very numerous anastomoses forming a primary network. Into the meshes of this net small arteries are given off at regular intervals, and these anastomose freely, forming a secondary cuboidal net of great regularity. From the threads of this network the arterioles branch off, generally at right angles to the muscle fibres and at very regular intervals (of about 1 mm in the warm blooded animal) and these arterioles split up into a large number of capillaries running along the muscle fibres and in the main parallel to them, but with numerous anastomoses, forming long narrow meshes about the fibres. The

> capillaries unite into venules intercalated regularly between the arterioles, and the whole system of veins reproduces and follows almost exactly that of the arteries."

For the most part, this classical description of the vascular architecture in skeletal muscle has been confirmed in rat spino-trapezius muscle (Zweifach and Metz, 1955; Stingl, 1970), in rat cremaster muscle (Grant, 1964; Grant and Wright, 1968; Baez, 1969; Smaje et al, 1970), in rhesus plantaris muscle (Hammersen, 1968) and in cat tenuissimus muscle (Eriksson and Myrhage, 1972).

I.3 The different types of fibres in skeletal muscle

Ranvier (1873, 1874a, b, c, 1880) in his studies on skeletal muscle, was the first to describe morphological differences between various muscles, pointing out that certain muscles, which appear red to the eye, have a "more abundant sarcoplasm and more numerous nuclei" than muscles having a more pale appearance. However, the existence of two types of muscle probably has been known since ancient times through preference for light and dark meat.

Later investigators noted a difference in number of mitochondria in various muscle fibres. Since 1950, advances in histological methods have led to morphological and histochemical "typing" of muscle fibres according to their metabolic pathway and characteristics of contraction.

Most authors agree that three distinct fibre types are present in muscle. However, at this time, no conventional system of

nomenclature for classifying the three types of fibres has been adopted (Brooke and Kaiser, 1970):

> "Studies on normal and pathological, striated muscle are increasingly clouded by inconsistencies in the definition of fiber types and lack of correlation between different systems of nomenclature."

A summary of the many proposed systems of classification is given in Table I. The system suggested by Peter et al (1972) seems most appealing for it describes each fibre by both its twitch characteristic and metabolic pathway; the three types are described as: (i) fast twitch glycolytic (classical white fibre), (ii) fast twitch oxidative-glycolytic (classical red fibre) and (iii) slow twitch oxidative (so-called intermediate fibre). The morphological and histochemical characteristics for the three types of fibres are given in Table II. A summary of the characteristics (Close, 1972) is as follows:

> "(a) Fast-twitch white fibers (have) relatively short isometric twitch contraction times, high activity of myosin ATPase, a well developed glycolytic enzyme system, low mitochondrial content and oxidative activity; these fibers fatigue rapidly. (b) Fast-twitch red fibers (have) relatively short isometric twitch contraction times, high activity of myosin ATPase, moderately developed glycolytic system, high total oxidative enzyme activity and many mitochondria; these fibers are much more resistant to fatigue than white fibers.

Table I+

Proposed methods of classifying the various types of fibres found in skeletal muscle.

Author(s) and Year	Types of muscle fibres		
Ogata and Mori (1958, 64)	white	medium	red
Dubowitz and Pearse (1960)	II	I	II
Stein and Padykula (1962)	A	B	C
Engel (1962, 70)	II	I	II
Romanul (1964)	I	III	II
Henneman and Olson (1965)	A	C	B
Olson and Swett (1966)	A	C	B
Padykula and Gauthier (1967)	white	intermediate	red
Kugelberg and Edström (1968)	A	C	B
Yellin and Guth (1970)	α	β	αβ
Brooke and Kaiser (1970a, b)	IIβ	I	IIA
Ashmore and Doerr (1971)	α-white	β-red	α-red
Burke et al (1971)	FF	S	FR
Barnard et al (1971)	fast twitch white	slow twitch intermediate	fast twitch red
Peter et al (1972)	fast twitch glycolytic	slow twitch oxidative	fast twitch oxidative-glycolytic

+ the data in this table have been taken from the papers of Close (1972), Peter et al (1972) and Peter (1973)

Table II

Morphological and histochemical characteristics of the various types of fibres in skeletal muscle

Characteristic	Fast glycolytic	Slow oxidative	Fast oxid-glyco.
mitochondrial content	small	intermediate	large
cytological localization	a few sarcolemmal aggregates	uniform throughout	many sarcolemmal aggregates
Z-line	narrow	intermediate	broad
fibre diameter	large	intermediate	small
neuromuscular junction	large, complex	intermediate	small, simple
granular size of particles	large	small	large
twitch time	fast	slow	fast
time for onset of fatigue	fast	?	slow
NADH diaphorase	low	intermediate	high
Malate dehydrogenase	low	intermediate	high
phosphorylase	high	low	high
PAS (glycogen)	high	low	high
α-glycerophosphate dehydrogenase	high	low	high or intermediate
hexokinase	low	high	intermediate
lactate dehydrogenase	high	low	intermediate
acid hydrolases	low	high	intermediate
succinic dehydrogenase	low	intermediate	high
and distribution	even network	even network	sarcolemmal
mitochondrial ATPase	low	intermediate	high
myofibrillar ATPase (pH9.4)	high	variable or low	high
pH sensitivity of myofibrillar ATPase activity	acid labile, alkali stable	acid stable alkali labile	acid labile, alkali stable
fermaldehyde sensitivity of myofibrillar ATPase activity	sensitive	?	stable
myoglobin content	low	high	high
gross colour	white	intermediate	red

> (c) Slow-twitch intermediate fibers (have a) low speed of contraction and [low] specific activity of myosin ATPase, poorly developed glycolytic enzyme system, high mitochondrial content and oxidative enzyme activities; these fibers show little or no fatigue."

To "type" muscle fibres histochemically, one must use quick-frozen, cryostat-sectioned material. As the facilities for preparing material in this way were not available, no attempt has been made to distinguish muscle fibre types in this study. However, when possible, data from the literature on fibre type distributions will be used for the comparison of results obtained for the various muscles used in this study.

I.4 The capillaries of skeletal muscle as seen in longitudinal section

The parallel nature of the capillary-muscle fibre geometry is readily apparent in Figure 4. It appears that one should be able to describe the vascular geometry in terms of capillary length. However, such measurements have been found to be both technically and conceptually difficult (Hammersen, 1968):

> "The variety and complicated three-dimensional distribution of muscle capillaries....might be responsible not only for the difficulties in gaining significant values for capillary length, quantity and other essential data for estimating the oxygen supply, but also for

Figure 4 - Longitudinal section of cat gastrocnemius muscle which has been injected with a silicone elastomer to outline the capillary supply. Each fibre represents a single multi-nucleated cell which is approx. cylindrical in shape and runs parallel to the other fibres. The capillaries supplying each fibre are located between the fibres and run, in the main, parallel to the fibres. The frequent anastomoses between the capillaries form a network surrounding each fibre.

Legend:	mf	-	muscle fibre
	c	-	capillary
	a	-	anastomosis
	n	-	nuclei

Scale: 1 cm ≡ 20 μm

> those in establishing a sufficiently exact and generally applicable prototype of these rather complex vascular arrangements.
> To begin with, even the term capillary length cannot be defined as precisely as would be desired... But if one chooses - and that is the general practice - the distance between afferent and efferent vessels, then their various directions to the muscle fibers already cause considerable differences in capillary length... Since in this calculation neither average number nor length of the intercapillary cross-anastomoses have been taken into account, which in our opinion could only be roughly estimated, nor the fact that the capillaries are not connecting arterioles and venules as straight tubes in the shortest way possible..., our data could only serve as a gross-orientation. Nevertheless, they point out that considerable variations of capillary length exist not only in <u>different</u> but also within the <u>same</u> muscle of <u>one</u> species..., so that innumerable measurements are required to gain statistically significant figures in this field."

The first measurements of capillary length were given by Spalteholz (1888) for rabbit muscle. The following passage recounts the extreme variation in capillary lengths encountered by Spalteholz in his study (translated by author).

> "In the length of capillaries one encounters moderate variations.... The mean length of a capillary in rabbit muscle without consideration of minimum and maximum amounts to 690 µm and it varies moderately symmetrically between 500 and 1000 µm but I can also measure ones of 430 µm and from 1000 to 1350 µm in length. The capillaries which separate in opposite directions from the same arteriole are mostly the same length or slightly different, I met however arterioles of which the capillaries going to one side were of 1080 and 1110 µm and to the other side 530 and 560 µm. Concerning the diameter and number of capillaries which go from a single artery, I am not yet in a position to make satisfactory details."

The first systematic examination of the capillary length was reported by Steudel (1938), who summarizes the state of the art at this time: "Bibliographical data on this measurement I have not yet found; it was therefore studied in several muscles, and thereupon expanded to different muscles", (translated by author). In regards to measuring the capillary length, Steudel states (translated by author):

> "The capillary net is readily recognized by its variability, and the multiplicity of the capillary net is not easily defined,"

Steudel defined the capillary length as the distance from the first point where a capillary branched to join another capillary (queranastomosen) to the final point of anastomosis before the

capillary joined a venule. These measurements did not include therefore, the distance from the arteriole to the first point of branching, nor the distance from the last anastomosis to the collecting venule.

Steudel recognized the importance of the cross-anastomoses: "There remains to be examined what ultimate meaning the cross-anastomoses have in the supply of blood to the musculature. It is not easy to comprehend them quantitatively." (translated by author). Steudel's measurements included both the distance between, and the breadth of the cross-anastomoses; he reports both values for the absolute number of 'loops' per capillary length, and normalized values per unit length of capillary (Appendix I).

Several authors (Rous et al, 1930; Smith and Rous, 1931; Nikiforova and Shoshenko, 1963; Smaje et al, 1970; Hammersen, 1970; Honig, 1970; Eriksson and Myrhage, 1972; Nikolov and Schumacher, 1972; Sabaev, 1973; Mankovska, 1973) have reported values (Appendix I) for capillary length in a number of species including man. The capillary length, in most instances, has been defined as the "straight line" distance between the points where the capillary branches off the arteriole and rejoins the venule.

Ivanova (1973) presents a systematic study of capillary length in a number of species. However, no definition of what length was actually measured is given by this author. It appears, from comparison with the other investigators, that the definition of capillary length used by Ivanova was similar to that used by Steudel (1938), ie. the "straight line" distance between the first and last capillary anastomoses.

I.5 The capillaries of skeletal muscle as seen in transverse section

The capillaries of skeletal muscle for the most part run along and parallel to the fibres (Figure 4). Sectioning the tissue transversely, ie. perpendicular to the long axis of the fibres, shows the fibres as large irregular polygons, and the capillaries as small dots (Figure 5).

Krogh (1919a, b, 1922) made use of this parallel nature of the capillaries in skeletal muscle in proposing an approach for quantitating the vascular supply, and hence oxygen transport, to the muscle fibres. It must be noted that each muscle fibre represents an individual muscle cell, and as such, a study of the relationship of capillaries and muscle fibres, provides quantitative information necessary for the understanding of supplying oxygen to a single cell. Krogh reasoned "that no serious error can be committed by supposing each capillary to supply oxygen, independently of all the others, to a cylinder of tissue surrounding it." Thus, in a transverse section, the area of tissue supplied by a single capillary (A_s) could be calculated by counting the number of capillaries (N_{cap}) in any known area of tissue (A_t) as $A_s = A_t/N_{cap}$. The radius of this area, termed the maximum diffusion distance (R_d) represents the farthest distance which oxygen must diffuse in order to adequately supply the tissue with its requirements and is calculated as $R_d = (As/\pi)^{1/2}$.

Krogh injected the muscles of a number of animals with a solution of gelatin and either india ink or Prussian blue. To compare the vascular supply between muscles, he determined the number of capillaries per mm^2 of tissue, ie. the capillary density (N_c/mm^2) and from this value calculated the maximum diffusion distance.

Figure 5 - Transverse section of cat gastrocnemius muscle which has been injected with a silicone elastomer to outline the vascular supply and stained with haematoxylin and eosin.

Legend:

mf - muscle fibre

c - capillary

n - nucleus

Scale: 1 cm ≡ 13 µm


n
c

In the years following Krogh's studies, most investigators followed the same procedure or a similar one with a few modifications; some controlled the injection pressure, others added vasodilators, and counted the number of capillaries by staining the erythrocytes present in the vessels. However, most investigators continued to use the capillary density as the index of skeletal muscle vascularization. A search of the literature has revealed 69 papers involving 334 determinations of the capillary supply in a variety of muscles from a number of species both at rest and under various experimental conditions. Of these 69 papers, 43 present values for the capillary density of the skeletal muscles in a number of species (Appendix II). Values as low as 72 vessels/mm^2 and as high as 5900/mm^2 have been reported for the capillary density of mammalian skeletal muscle, an increase of approximately 82 times! Several authors, feeling that the extreme variation observed in the capillary density was the result of biological variation or the particular method employed, suggested that the vascular supply in skeletal muscle should be "tied" to a common denominator, the fibre density (and thus mean fibre size).

Stoel (1925) was the first to present values for both the capillary and fibre densities. During this investigation, he noted that the "fibres of red muscle are thick and less numerous, whereas those of white muscle are thin and more numerous"; however, he does not comment on the substantial effect which this difference in fibre size would have on the observed capillary density. Jores (1928) presented a new method of measuring skeletal muscle vascularization

by taking the quotient of the capillary and fibre densities. This new index of capillary supply was termed the capillary: fibre ratio (C/F ratio). The range of C/F ratios reported for mammalian muscle since 1928 is 0.30 to 4.00 capillaries per fibre, a difference of approximately 13 times (see Appendix II for individual values).

Bosiger (1950) reported that the number of capillaries "surrounding" each fibre in the skeletal muscle of birds ranged from one to six, the exact value being dependent on the type of muscle fibre examined. Valdivia and co-workers (1956, 1958, 1960) found that in guinea pigs, the mean number of vessels around each fibre varied from 6.4 to 9.1 and was a function of the muscle used for study. Several other authors (Henneman and Olson, 1964; Romanul, 1965; Taylor et al, 1973; Asmussen and Kiessling, 1974) have commented that in both mammals and amphibians the number of capillaries surrounding a fibre seemed to vary from zero to eight. More recently, mean values of 3.9 to 5.3 for guinea pig (Mai et al, 1970) and 3.5 to 3.8 for cat (Eriksson and Myrhage, 1972a, b) have been reported; these values were found to depend on the type of fibre being examined.

From the viewpoint of oxygen diffusion, this last approach to studying the relation of the capillaries to the muscle fibres is most appealing. Many investigators in the field of gas exchange have focussed, almost exclusively, on the supply of oxygen from a single capillary or else from a matrix of capillaries embedded in a mass of homogeneous tissue. However, each muscle fibre represents an individual "consumer", and it seems appropriate to look at oxygen supply from the consumer's point of view. In other words, how many vessels are needed around the circumferehce of a muscle fibre for

provision of an adequate supply of its O_2 requirements?

I.6 Review of methods used in demonstrating the capillaries in skeletal muscle

In his initial studies on the capillary supply of the fibres in skeletal muscle, Krogh injected the muscles of animals with "a suspension of particles which would be evenly distributed in the blood and by their presence indicate the capillaries through which the blood had been flowing." The suspension was composed of gelatin and either India ink or Prussian blue and the injection was "directly through a muscle artery, tying as far as possible all the arterial anastomoses and employing a high pressure. When practicable I have finally tied the vein and set up a considerable pressure before tying the artery" (Krogh 1919a).

Since that time, several other methods have been employed for demonstrating the number of capillaries in skeletal muscle. These new methods include:

(1) variations of Krogh's original perfusion technique,

(2) staining the erythrocytes or endothelial nuclei of the capillaries,

(3) histochemistry, ie. staining specifically for an enzyme (alkaline phosphotase) or a component (mucopolysaccharide by periodic acid-Schiff reaction) of the capillary wall,

(4) direct visualization, and

(5) electron microscopy.

The restrictions placed on each method will be discussed below.

(1) Perfusion studies

A wide variety of substances have been used to demonstrate the capillaries in skeletal muscle. India ink in gelatin (from Krogh) has been the most popular although tryphan-blue (Jores, 1928), graphite (Perry, 1930), methylene blue (Vannotti and Magiday, 1934), Congo red (Vannotti and Magiday, 1934), Berlin blue (Vannotti and Gukelberger, 1935) and latex (Smith and Giovacchini, 1958) have been suggested as an "ideal" injection medium. The main problem with injection methods is one of obtaining complete filling of the vascular bed. Krogh (1919a) recognized the problem:

> "I intended to test this deduction on a series of injection preparations of muscles from a number of animals, but I have had to reduce the programme very considerably on account of the unexpected difficulties met with in obtaining complete injections of muscular tissue."

To overcome this difficulty a number of 'improvements' have been made to Krogh's procedure, including the use of several potent vasodilators (amyl nitrite, papaverine), electrical stimulation of the muscle or pre-washing of the muscle with warm (40°C) Ringer's solution. However, no single improvement has consistently produced a high level of perfusion of the vascular bed. Several investigators have noted a pattern of vascular filling similar to that described by Martin and co-workers (1932):

> "A striking observation, and one that seems to us probably significant, is that in all these muscles fasciculi that showed no injected capillaries, or at most a very few, were interspersed among well-injected fasciculi."

This pattern may be the result of: (i) interaction of India ink (or dye particles) with the vessel walls or with each other (Hartman et al, 1929).

> "Upon investigation it was found that clumps of particles were caught at the junction of the arteriole and capillaries so that they did not enter the latter. After many attempts we decided that the ink injection method did not give a true picture of the open capillaries."

(ii) a higher viscosity of the injection material than that of blood (especially in the case of latex materials), (iii) a high interfacial surface tension which may lead to selective distribution of the injection material, or (iv) a control system regulating capillary perfusion which has an 'on-off period' greater than the time taken for injection and/or is not affected by either the vasodilator substances used, or electrical stimulation.

(2) Staining erythrocytes or endothelial nuclei

Staining of erythrocytes was made popular by Petrén and Sjöstrand (1934). Stains used for demonstrating the erythrocytes include benzidine and orthotoluidine (Sjöstrand, 1934), haematoxylin and eosin (Lindgren, 1935), modified method of Lepehne (Becker, 1955) and acid fuchsin (Valdivia et al, 1960). However, Hakkila (1955) points out that the weakness of such a method

> "...lies in the fact that some parts of the capillaries may only contain plasma or some of the capillaries may be open sufficiently to permit plasma to pass through."

(3) Histochemical methods

Several methods of demonstrating the capillaries of skeletal muscle have been developed which are based on a reaction with either an enzyme or a structural component of the capillary wall. Basically three methods have been used: (1) alkaline phosphatase (Gomori, 1939), (2) adenosine triphosphatase (Wachstein and Neisel, 1957) and (3) periodic acid-Schiff reaction (Hecht, 1958).

These methods appear to be vastly superior to the other methods employed but they also have limitations. Beckett and Bourne (1958) state in regard to alkaline phosphatase: "It is perhaps interesting that only a small proportion of the blood vessels present [in skeletal muscle of both rat and human] give this reaction and.... It may be that only a small proportion of the blood vessels contain the enzyme permanently...". This opinion is contrasted by that of Romanul and Bannister (1962), who report:

> "The large arteries showed no staining in their walls. Primary branches of such arteries, however, with luminal diameters of 25 micra or less, displayed moderately intense staining in the endothelium starting abruptly at their point of origin from the larger vessel and gradually decreases distally.... Secondary branches originating from an unstained part of the primary branch showed the same pattern of enzymatic activity of endothelium starting abruptly at their point of origin and fading distally....In these branches, however, the staining was more intense, and extended throughout the length of the vessel. Capillaries originating from these branches stained with the same intensity as the distal portion of the secondary branch. The capillaries had rather uniform enzymatic activity even at their points of branching. There was a slight decrease in their intensity of staining near the venous end".

Thus it seems that the existence of alkaline phosphatase in the vessel wall is quite variable. This view has also been expressed in regards to the periodic acid-Schiff (P.A.S.) reaction. Rakusan (1971) stated that "this impregnation [periodic acid-Schiff] is not thorough in organisms at the early stage of development."

Both the alkaline phosphatase and ATPase methods necessitate the use of quick-frozen cryostat sections and require careful handling during processing if sufficiently good results are to be obtained.

(4) Direct visualization

This method, which has been used by Honig and his co-workers (1970) for some time, has two disadvantages: (1) only surface capillaries can be visualized, making any interpretation of the three-dimensional geometry impossible and (2) only certain exterior muscles can be analyzed by this method if excessive trauma to the tissue is to be avoided.

(5) Electron microscopy

Using electron microscopy may be the best method for obtaining the 'anatomical maximum' number of capillaries in skeletal muscle. Several investigators (Eisenberg et al, 1974; Eisenberg and Kuda, 1975; Casley-Smith et al, 1975) have started such analysis. The main problems with this method are the cost of the apparatus, the time required to achieve the desired results and the rather limited field of view to which one is restricted.

Perhaps the state of the art is best described by Hammersen and Appell (1976):

> "...There is still no laboratory tool to demonstrate with absolute reliability the total number of capillaries actually existing in a tissue as non-perfused microvessels cannot be visualized with any of the employed techniques, not even by means of histochemical methods...."

I.7 Review of indices used in describing the vascular supply in skeletal muscle

Determinations of capillary density have been found to be inadequate in comparing the vascular supply of different muscles. Firstly, muscle fibres vary in size, and as the capillaries are located only around the periphery of a muscle fibre (Figure 5), this variation in mean fibre diameter from muscle to muscle (and even in the same muscle from two animals of the same or different species) has a definite effect on the measured capillary density. Secondly, the procedures routinely used in histological preparation invariably lead to some degree of tissue shrinkage during processing. Many investigators (including the author) have not made any attempt to quantitate tissue shrinkage, thus rendering a comparison of the capillary density in different muscles virtually impossible. Examination of Appendix II reveals a wide disparity in reported values. For example, values of 2341 and 379 vessels/mm^2 have been reported for gastrocnemius muscle of cat. This represents more than a six-fold difference! It seems impossible that either tissue shrinkage and/or variation in fibre size could account for such differences as found in the literature (Appendix II).

In reviewing the methodology of each author, it became apparent that the discrepancy may have been related to the time period in which the study was undertaken. Indeed, values of capillary density from the period prior to 1950 are approximately twice as great as those reported in the years following 1950 (Appendix II). This

difference strongly suggests inherent errors in technique during one time period or the other. To test this hypothesis, the data were separated into two groups, namely pre- and post-1950.

A separation point was made at 1950 for two reasons: (1) few reported values appeared in the literature from 1938 to 1950 and (2) new methods for histological processing were developed after 1950 (including cryostat sectioning and histochemical identification of vessels).

These values were then sub-divided into two 'physiological' groups based on the vascular state of the muscle. The reports, in which the authors induced maximal patency of the vessels by chemical, mechanical or electrical stimulation of the vascular bed, were termed vasodilated muscle. Any other values were treated as resting muscle. The present study was undertaken to ascertain a baseline value for the anatomical maximum of the vascular supply in skeletal muscle. It seems reasonable, therefore, to discuss in detail only the values for vasodilated muscle, in which it was thought that the total vascular bed was identified.

The results of analyzing the reported values for both resting and vasodilated muscle are presented in Table III. It should be noted that: (1) the capillary density reported for vasodilated muscle is greater than that for resting muscle (regardless of the time period); (2) in either time period, the fibre densities are not significantly different; (3) the capillary/fibre ratios, reflecting the differences in capillary density, are also greater for vasodilated muscle; (4) the pre-1950 values for both the capillary and fibre densities are much

greater than the corresponding values post-1950. It appears that the pre-1950 values for both capillary and fibre density are unexplainably large.

When the present study began the muscle sections were routinely stained with haematoxylin and eosin (H & E). However, counting the capillaries in sections stained in this way was exceedingly difficult as the darkly stained nuclei, also peripherally located, were easily confused with the injected capillaries. It seemed possible that past investigators may have experienced a similar problem. A re-examination of the reported values according to methods employed proved quite illuminating.

Each study was placed in one of three categories: (1) haematoxylin was definitely part of the method, (2) haematoxylin was definitely not used and (3) insufficient details of the method were presented to allow placement in either category 1 or 2. The results of analyzing the reported values in this manner are presented in Table IV. Several new facts come to light: (1) the capillary density in those studies where haematoxylin was part of the method is definitely greater than that determined by other means; (2) there is little difference between any of the values for resting and vasodilated muscle determined by methods not involving haematoxylin.

Comparison of Tables III and IV reveals that the reported values appear to be indicative of the time period in which the study was done and of the method employed in the study. Statistical analysis of these results (Tables V and VI) shows that indeed the reported values are well correlated with the time period and method.

Table III

Summary of pre- and post-1950 values reported for resting (R) and vasodilated (V) muscle

Time period & vascular state		Capillary density $\bar{x}$	c.v.	n	SE	Fibre density $\bar{x}$	c.v.	n	SE	Capillary/fibre ratio* $\bar{x}$	c.v.	n	SE
pre-1950:	R	1615	0.41	19	150	1161	0.75	19	199	1.65	0.41	19	0.15
	V	3053	0.55	5	746	1689	0.73	5	551	2.05	0.35	5	0.32
	R/V	0.53				0.69				0.80			
post-1950:	R	1108	0.59	67	80	660	0.61	67	49	1.70	0.39	67	0.08
	V	1484	0.55	16	205	611	0.22	16	34	2.35	0.49	16	0.29
	R/V	0.75				1.08				0.72			
pre/post:	R	1.46	>0.71			1.76	>0.64			0.97	> 1.12		
	V	2.06				2.76				0.87			

$\bar{x}$ - mean

c.v. - coefficient of variation

n - number of observations

SE - Standard error of the mean

* - mean of the ratios

Table IV

Summary of values reported for resting (R) and vasodilated (V) muscle for methods of which haematoxylin was a part (H) and those of which it was not (no H)

Method employed & vascular state	Capillary density $\bar{x}$	c.v.	n	SE	Fibre density $\bar{x}$	c.v.	n	SE	Capillary/fibre ratio* $\bar{x}$	c.v.	n	SE
H: R	1479	0.28	27	79	801	0.43	27	66	2.01	0.32	27	0.13
V	2642	0.44	10	366	1123	0.90	10	318	2.96	0.33	10	0.31
R/V	0.56				0.71				0.68			
noH: R	1060	0.59	32	110	692	0.48	32	59	1.49	0.35	32	0.10
V	1024	0.56	6	234	653	0.29	6	77	1.50	0.37	6	0.23
R/V	1.04				1.06				0.99			
H/noH: R	1.40	>0.54			1.16	> 0.67			1.35	> 0.69		
V	2.58				1.72				1.97			

$\bar{x}$ - mean

c.v. - coefficient of variation

n - number of observations

SE - standard error of the mean

* - mean of the ratios

Table V

Statistical analysis of capillary density according to time period and method employed

Capillary density*	pre-1950	post-1950	H employed	no H employed
pre-1950	-	< .01	.40	+
	-	< .01	.50	+
post-1950	.01	-	+	>.50
	<.01	-	+	.25
H employed	.40	+	-	<.01
	.50	+	-	<.001
no H employed	+	>.50	<.01	-
	+	.25	<.001	-

* in each case, the value for resting muscle is presented first.

\+ t-test not done.

Table VI

Statistical analysis of fibre density according to time period and method employed.

Fibre density*	pre-1950	post-1950	H employed	no H employed
pre-1950	-	<.001	.60	+
	-	.001	.30	+
post-1950	<.001	-	+	>.50
	.001	-	+	.50
H employed	.60	+	-	<.01
	.30	+	-	.25
no H employed	+	>.50	<.01	-
	+	.50	.25	-

* in each case, the value for resting muscle is presented first.

\+ t-test not done.

As stated earlier, new methods of histological processing were developed after 1950, including the use of frozen sections using the cryostat, and histochemical identification of the capillaries. In fact, most of the post-1950 values for capillary supply were obtained using frozen sections and either incubation for the enzyme alkaline phosphotase or the PAS reaction. Therefore it seems likely that many of the pre-1950 values are "inflated" because the early investigators confused the darkly stained nuclei (the result of staining with haematoxylin) with the dark appearing India ink-filled capillaries.

The analysis of the reported fibre densities (Tables III and IV) show that the pre-1950 reports yield consistently higher fibre densities (Table VI). These higher values are undoubtedly the result of the great amount of shrinkage incurred in processing the sections by standard histological procedures. As a result of using frozen sections, the post-1950 investigators found less shrinkage and more consistent values (compare the coefficients of variation).

The difficulties with regard to the measurement of capillary density have emphasized the need for a different approach to the quantitative description of the vascular supply of skeletal muscle fibres. Some authors (see Appendix II) have used the capillary/fibre ratio (C/F ratio), ie. the total capillary count per unit area (usually $1 mm^2$) divided by the total fibre count in the same area of tissue. This measurement eliminates both the variable effect of tissue shrinkage, and any differences in mean fibre size, but, is inherently dependent on the particular arrangement of the capillaries and fibres

in the section being observed, and has been found to be quite sensitive to variations in the size of the field of view being used for counting.

The dependence of the capillary/fibre ratio (C/F ratio) on the type of vascular arrangement being studied is illustrated by Figures 6a-d, and summarized in Table VII. Each fibre in arrangement 6a has four capillaries distributed around its periphery, and each is located between two fibres, that is to say, each capillary is "shared" by two fibres (SF = 2). In an infinite array of this description the C/F ratio will be two. The three parameters are related by the fact that the determination of the number of capillaries around each fibre (N_{VF}) will include each vessel more than once; in fact, each capillary will be counted in proportion to its sharing factor. Thus the number of capillaries around a fibre (N_{VF}) equals the product (C/F ratio)·(SF). The values for N_{VF}, SF and C/F ratio for each of the arrangements 6a-d are presented in Table VIII. It is quite evident that the C/F ratio can vary as much as 200% (ie. from 1.0 to 3.0) depending on the particular arrangement of capillaries and fibres.

It is also possible to "bias" the C/F ratio either by "squeezing" into the field of view all the capillaries possible, or by neglecting the parts of those fibres not wholly within the field of view. The two arrangements in Figure 7 illustrate the effect which varying the size of the field of view has on the observed C/F ratio. The two arrays are expanded examples of Figures 6a and b, and the circles represent the boundaries of imaginary fields of view, each drawn from a common centre. The diameter of these fields of view ranged from two to 16 fibre diameters, a difference in area of 64

Figure 6 - Several models which have been proposed for describing the relationship of capillaries and muscle fibres in transverse sections of skeletal muscle. The black dots denote capillaries, and the stippled circles represent muscle fibres. The relationship of capillaries to muscle fibres is quite different for each arrangement. The characteristics of each model are:

(a) square array: $N_{VF} = 4$; C/F ratio = 2; SF = 2

(b) square array: $N_{VF} = 4$; C/F ratio = 1; SF = 4

(c) hexagonal array: $N_{VF} = 6$; C/F ratio = 2; SF = 3

(d) hexagonal array: $N_{VF} = 3$; C/F ratio = 1; SF = 3

Legend:

N_{VF} - number of capillaries around each fibre

C/F ratio - ratio of number of capillaries to number of fibres in a given area

SF - number of fibres sharing one capillary

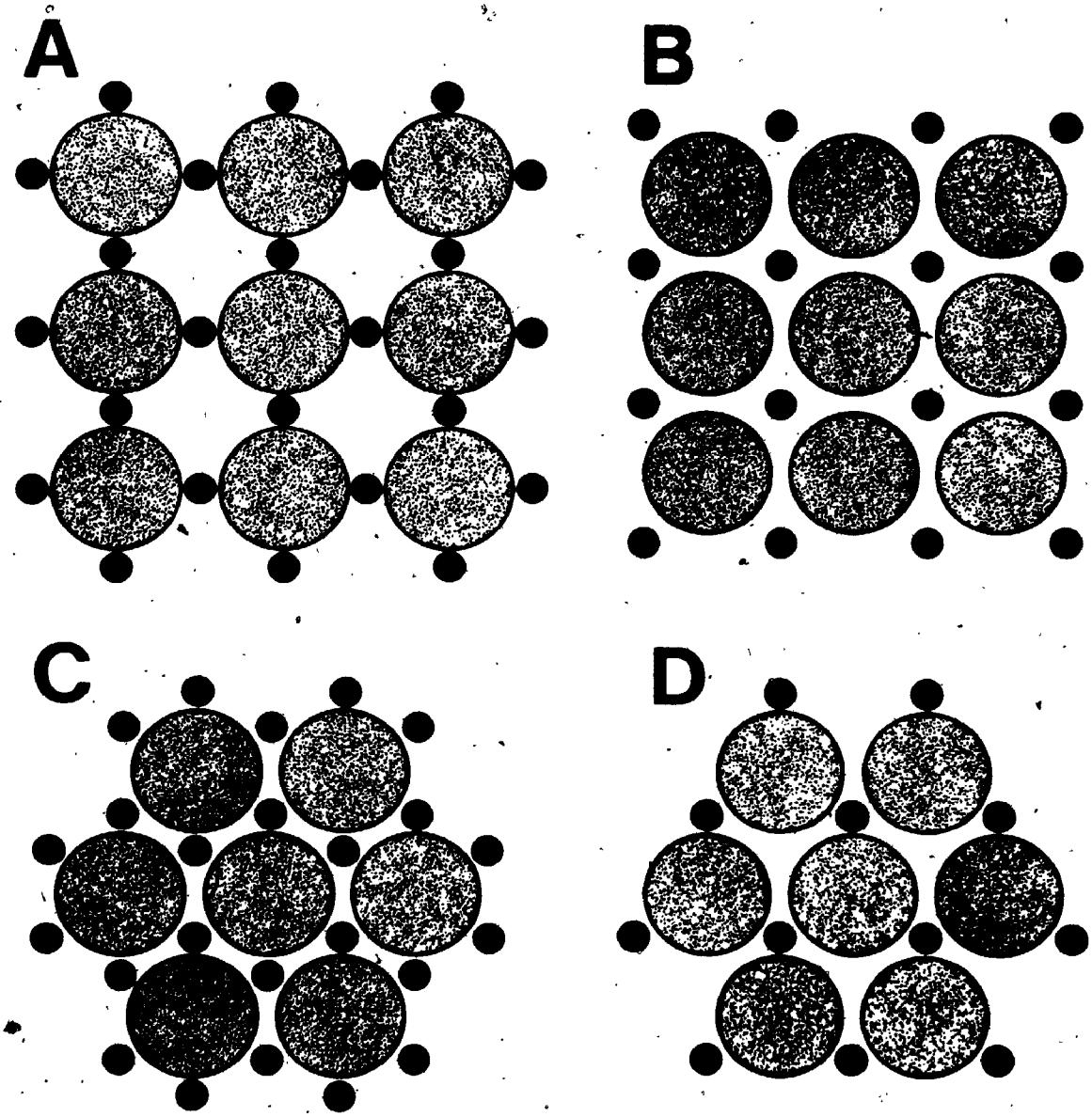

A
B
C
D

Table VII

Descriptive parameters for the arrangements presented in Figures 6a-d

Arrangement	N_{VF}	SF	C/F ratio	Array type
a	4	2	2	square
b	4	4	1	square
c	6	3	2	hexagonal
d	3	3	1	hexagonal

Table VIII

Dependence of calculated C/F ratio on the type of vascular arrangement being observed

Arrangement[1]	Capillaries[2]	Fibres[2]	C/F ratio[3] calc.	C/F ratio[3] act.	Increase[4] (%)
a	24	9	2.67	2.0	33.5
b	16	9	1.78	1.0	78.0
c	24	7	3.43	2.0	71.5
d	30	7	4.29	3.0	43.0

1. refers to the arrangements illustrated in Figures 6a-d

2. the capillaries and fibres were counted as if the figure represented one field of view

3. calc. = calculated from capillary and fibre counts
   act. = actual or limiting value for an infinite array of this description

4. the percentage increase in calculated C/F ratio if such a limited field of view is used for counting

times. The C/F ratio was determined for each field of view and compared with the known value for such an arrangement (Tables IX and X). In each case, it is readily apparent that the C/F ratio decreases, toward the known values of 2.0 and 1.0 as the diameter of the field of view is increased. This is to be expected, for increasing the size of the field of view leads to a closer approximation of the infinite array from which the optimum value is derived. This fact has been known for some time by haematologists. When performing blood cell counts, all cells within the given area are counted and to this is added one-half of the cells lying on the boundary of the sample area. If one follows a similar procedure when determining the C/F ratio, the results are much more consistent and appear to be independent of the size of the field of view (Tables IX and X). As yet, no report concerning the effect of the diameter of the field of view on the observed C/F ratio has been found. This simple analysis indicates that counts of capillaries in fields of view less than ten fibre diameters wide should be corrected by subtracting one-half the number of capillaries observed on the edge of the field of view from the total count.

Rakusan (1971) feels that the F/C ratio (ie. 1/C/F ratio) should not be used because it "ignores the size of the individual muscle fibres". He suggests that a "more suitable index of the capillary supply of the tissue is the diffusion distance... defined as the mean half distance between two capillaries in a cross section of the muscle". However, as shown in Figure 8, there are areas of tissue, (hypoxic zones) which will not be supplied with O_2 unless the area of tissue supplied by each capillary overlaps with several others. Therefore, to use the diffusion distance necessitates: (1) compensation

Figure 7 - Schematic illustrating the error which may occur when measuring the capillary/fibre ratio in small fields of view; A and B refer to arrangements A and B of Figure 6. The small black dots denote capillaries; the larger solid circles depict muscle fibres and the dotted circles represent fields of view from four to 20 fibre diameters across. The computed values of C/F ratio as a function of field diameter are presented in Tables IX and X (see overleaf).

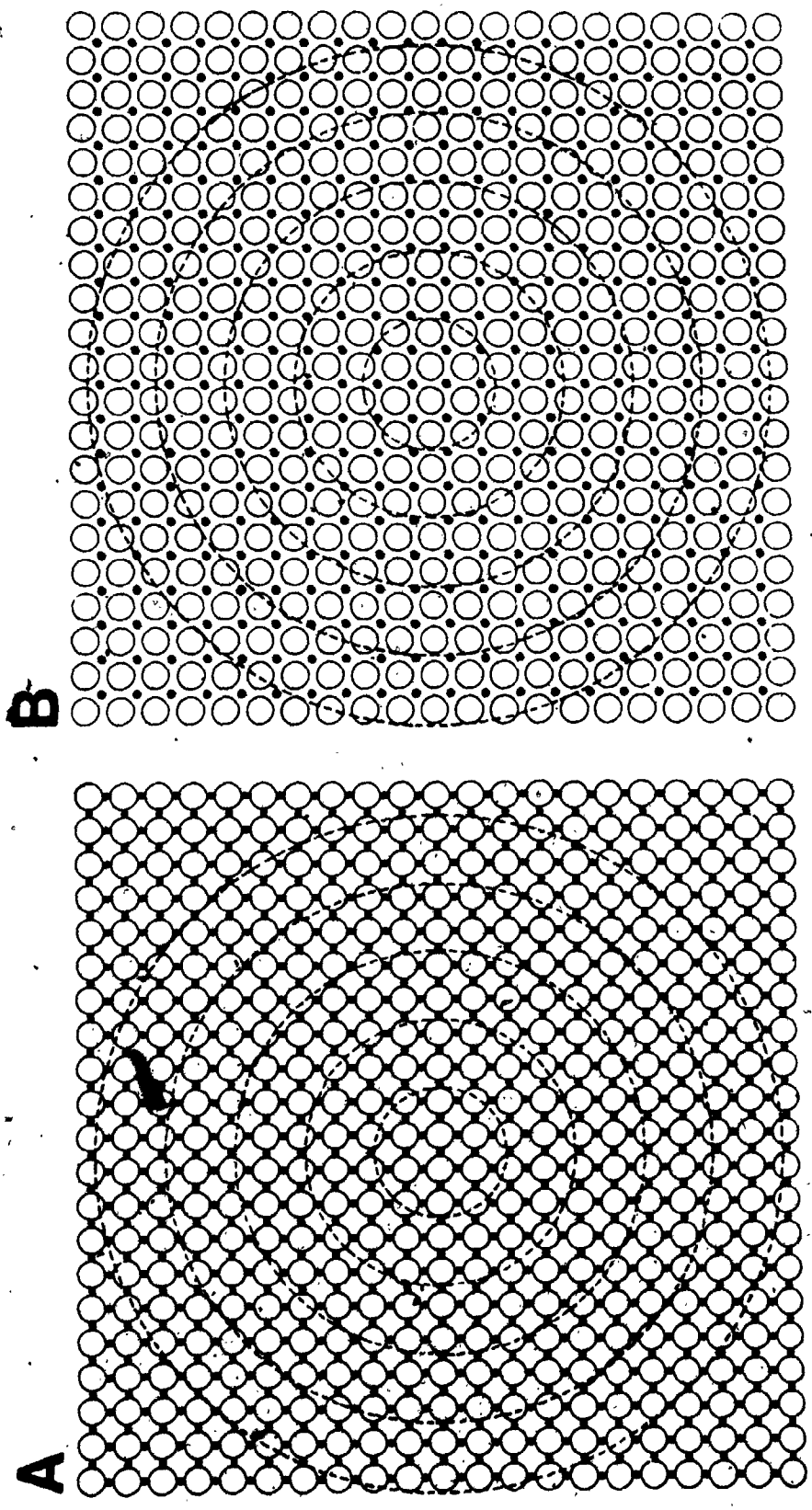

B
A

Table IX

C/F ratio measurements as a function of the diameter of the field of view for array 7a

Field of view (in fibre diameters)	C/F ratio[1]	Increase[2] (%)	Corrected[3] C/F ratio	Increase[2] (%)
4	2.27	+ 13.5	2.09	+ 4.5
8	2.11	+ 5.5	2.02	+ 1.0
12	2.05	+ 2.5	2.01	+ 0.5
16	2.06	+ 3.0	2.01	+ 0.5
20	2.04	+ 2.0	2.01	+ 0.5
Mean ± SE	2.11 .04		2.03 .02	

1. calculated from counts of capillaries and fibres within the boundaries of each 'field of view'
2. increase in C/F ratio as compared with the ideal value of 2.0
3. corrected by adding one-half the 'boundary' count to the 'interior' count

Table X

C/F ratio measurements as a function of the diameter of the field of view for array 7b

Field of view (in fibre diameters)	C/F ratio[1]	Increase[2] (%)	Corrected[3] C/F ratio	Increase[2] (%)
4	1.45	+ 45.0	1.27	+ 27.0
8	1.22	+ 22.0	1.09	+ 9.0
12	1.07	+ 7.0	1.02	+ 2.0
16	1.04	+ 4.0	1.01	+ 1.0
20	1.03	+ 3.0	1.01	+ 1.0
Mean	1.16		1.08	
± SE	.08		.05	

1. calculated from counts of capillaries and fibres within the boundaries of each 'field of view'

2. increase in C/F ratio as compared with the ideal value of 1.0

3. corrected by adding one-half the 'boundary' count to the 'interior' count

of the radius of diffusion by a distance equal to one-half the diameter of the lethal corner, or (2) making the assumption that each capillary supplies an area of tissue which is hexagonal in shape (Thews, 1960). The equation for diffusion distance (R) in this case is as follows:

$$R = \left[\frac{2A}{3\sqrt{3n}}\right]^{1/2}$$

where R equals the side of the hexagon

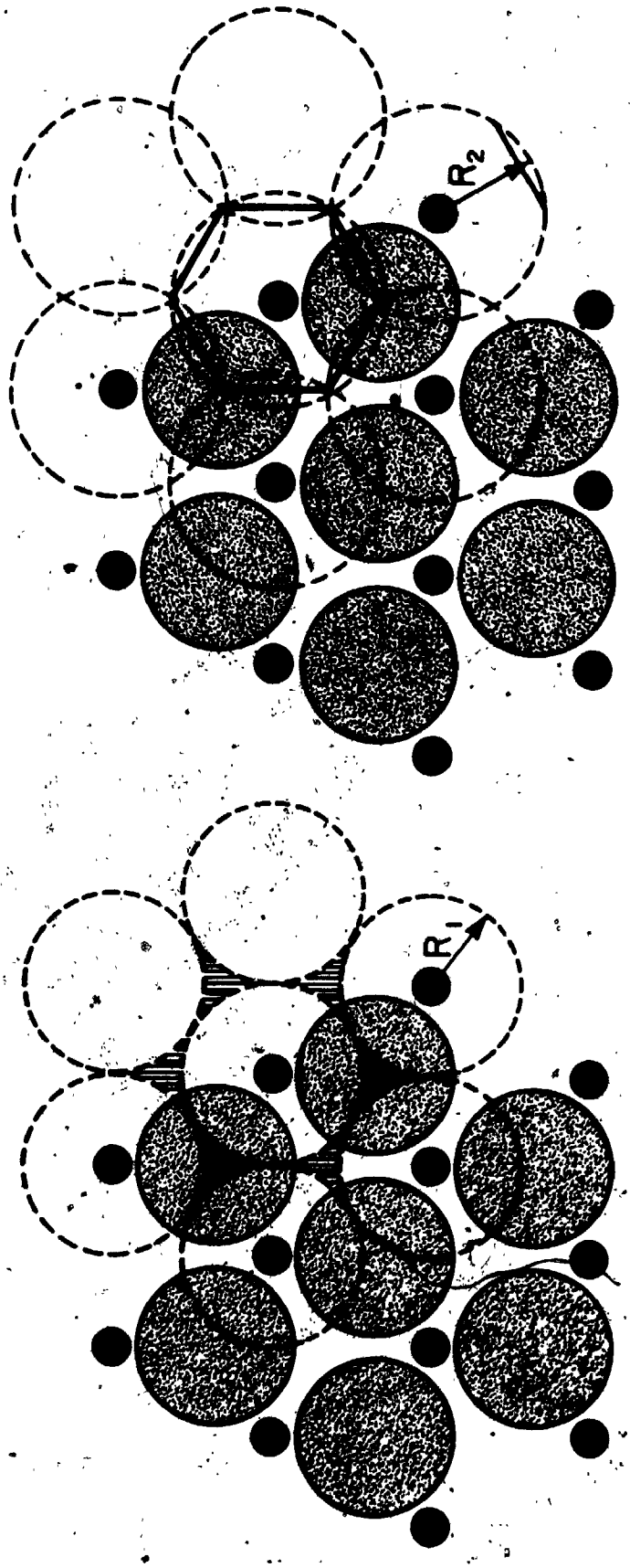
A equals size of the area

n equals the number of capillaries counted in the area.

Some investigators (including the author) have presented values for the number of vessels around each fibre (N_{VF}); however, this index of capillary supply also has its disadvantages. The primary disadvantage is the possibility that two fibres of significantly different size may have the same number of vessels supplying them, which implies that either one fibre is over-supplied with O_2, or the two fibres have a significantly different metabolic rate. Such a result may be advantageous, however, in that it indicates a consistency in regards to describing the vascular supply to the fibres of skeletal muscle, whatever may be the underlying cause, not seen with any of the other indices. Being unaffected by tissue shrinkage, vascular arrangement or mean fibre size, N_{VF} could lead to a more clear understanding of the factors underlying vascular supply to tissue.

In calculating either capillary density or the C/F ratio, miscounting and/or misfilling the vessels leads to significant errors

Figure 8 - Two concepts of the diffusion distance. Left: the classical Krogh cylinder approach in which the material diffuses radially outward to a distance (R_1) equal to one-half the distance between two actively perfused capillaries. As the regions of supply do not overlap, certain areas of the tissue (shaded areas) will not be adequately supplied with oxygen. Right: a modification by Thews (1960) in which the diffusion distance (R_2) is equal to the radius of the circumscribed circle of each hexagon of tissue, thus providing adequate supply to all areas of the tissue (modified from Rakusan, 1970).


R2
R1

which are often compounded when the observed count (usually quite small) is converted to one square millimetre: (count)·($1mm^2$/area of the field of view). This possibility can be avoided by using a larger field of view for analysis. However, if the field is too large (ie. very low magnification) accuracy is again decreased because there is a greater chance of miscounting the capillaries. Naturally, N_{VF} is also quite sensitive to misfilling of the capillaries (miscounting is unlikely). However, if a sufficiently large number of counts are made ($n > 300$; Snedecor, p 501), the loss of one capillary, ie. one incorrect count, is "dwarfed" by the number of correct counts. In the present study, in <u>each</u> experiment N_{VF} was determined for $n > 500$ fibres.

This analysis of the reported values in the literature (Appendix II) has shown that: (1) methods used prior to 1950 (ie. perfusion studies or erythrocytes staining procedures) underestimate the capillary density as compared to the fibre density. This is undoubtedly the result of inadequate filling of the vascular bed in the case of perfusion studies, and inconsistent identification of capillaries due to "plasma gaps" between erythrocytes in the case of the red cell staining method. (2) The histological procedures employed in the foregoing methods resulted in substantial tissue shrinkage, which most likely varied quite considerably from muscle to muscle, or even from section to section. (3) Those studies in which haemotoxylin staining followed perfusion of the vascular bed, have overestimated the number of capillaries as compared to the fibres. It is the contention of the author that the nuclei of muscle and/or

connective tissue have been confused with the injected capillaries by the investigators using this protocol. (4) Each index describing the vascular supply of skeletal muscle has inherent disadvantages; however, the number of vessels found around the periphery of each cell (N_{VF}) appears to be superior to the other indices (provided a large number of counts are made).

I.8 The need for measurements on the geometric relation between capillaries and muscle fibres

Although the "hair-like vessels" (capillaries) were discovered as early as the 17th century, research on their importance was slow in starting for both methodological and practical reasons. Krogh (1922) summarizes the feelings of his time:

> "We have a propulsive organ: the heart; a distributing organ: the system of arteries; an organ for interchange of substances between the blood and the tissues: the capillaries; an organ for collecting the blood and carrying it back to the heart: the venous system. It is evident that the organs of propulsion, distribution and carrying back are all subservient to the function of exchanges carried out in the capillaries, and though, of course, each of the great organs is absolutely necessary for the functioning of the whole, it will be difficult to challenge the proposition that the capillaries constitute the most essential part of the whole circulating system. It is a little strange, therefore, to find that, far from being a favourite subject for anatomical and physiological research, the capillaries have

> been neglected to an extraordinary degree. Though about two hundred years have passed since the capillaries were discovered you can find them dealt with in a few lines in quite modern text-books on physiology, and the references to their structure, given in text books of histology, are likewise of the most summary character."

V

Krogh was interested in the role of the capillaries in the supply of oxygen to tissue.

> "In order to solve this general problem or the more modest part of it..., the first thing to do must be to find out about the number, distribution and surface of capillaries in those tissues in which we are interested. For information of this kind we naturally turn to the anatomical literature, but I regret to have to say that in the main we are disappointed. We cannot find there that quantitative information which we require. We will find a certain number of papers in which the distribution of capillaries in different tissues is described, and we will find numerous figures illustrating the distribution, but the capillaries have practically never been counted,..."

Why, when numerous figures and illustrations of the capillary bed exist, have the number, distribution, length, etc. of capillaries not been determined? Krogh postulates that the early investigators were not trained in making such quantitative measurements.

> "...the illustrations from which at least approximate countings could in many cases be made, are as a rule deficient in that one respect which is for our purpose the most essential: the magnification is not given at all, or is given in such an ambiguous way that it is impossible to be sure whether it is the actual magnification of the figure published, the magnification of the original drawing (which is usually arbitrarily reduced in reproduction) or merely the magnification of the microscope employed, which is meant."

However, even today, these basic anatomical measurements have not been undertaken. As noted by Krogh (1922), this is due in part to the highly complicated nature of the capillary bed.

> "It is only right to add, further, that in most tissues the arrangement of capillaries is so complicated that the difficulties in the way of even approximate measurement are very formidable."

For this latter reason, Krogh chose to study the oxygen supply to skeletal muscle and the means by which the supply is regulated.

> "My problem was the supply of oxygen to the fibres of striped muscle, the mechanism by which it was brought about and, especially, how it could be regulated. The oxygen is presented to muscle fibres in the blood running through the capillaries which traverse the tissue. By whatever means the transport of oxygen from the capillaries to the

> muscle elements is brought about, it is clear that facility of transport must be related to the number and distribution of the capillaries and related, further, to the permeability for oxygen of the capillary walls and the tissues themselves. An essential part of my task must therefore be to try and obtain information on these points."

For this pioneering work on the oxygenation of tissue, August Krogh was awarded the Nobel prize in physiology in 1920, and laid the foundation for further studies on the capillary architecture in tissue. However, developments since the time of Krogh have been few, the problems many, as stated Martini and Honig (1969):

> "These shortcomings reflect, in large part, lack of anatomical data, not only as to the number of capillaries, but also their arrangement and distribution.... It would appear that almost half a century after Krogh's plea for "a quantitative anatomy" physiologists must obtain facts about capillary number, distribution, and arrangement by direct observation if further theoretical progress is to be made."

When Krogh made his study of oxygen transport to tissue, he was armed with a great deal of ingenuity, a few well conceived experiments and a simple mathematical relation. Today we have a vastly increased knowledge of mathematical methods for use with high speed digital computers as well as improved histological techniques;

however, progress in the field of oxygen transport to tissue is still limited, as explained by Honig and Bourdeau-Martini (1973):

> "We conclude that progress in O_2 transport is chiefly limited by lack of knowledge of parameters. In view of advances in mathematical modelling techniques..., it seems likely that exciting new insights can be expected from theoreticians only if experimentalists can provide the anatomical and physiological facts."

Over 50 years ago, Krogh made a plea for "quantitative anatomy", yet today the knowledge of vascular geometry in most tissues is of a "meagre and unsatisfactory kind". To express such relationships in numbers and advance our understanding of them to the "state of science" is indeed a challenge to us all.

"In scientific investigation, minutiae of method are of the highest importance. The happy choice of an animal, an instrument constructed in some special way, one reagent used instead of another, may often suffice to solve the most abstract and lofty questions. Every time that a new and reliable means of experimental analysis makes its appearance, we invariably see science make progress in the questions to which this means of analysis can be applied. On the contrary, a bad method or defective processes of research may cause the gravest errors, and may retard science by leading it astray. In a word, the greatest scientific truths are rooted in details of experimental investigation which form, as it were, the soil in which these truths develop."

(Claude Bernard, 1865)

II. METHODS AND ANALYSIS

II.1 Transverse section studies

Analysis of the capillary geometry as seen in transverse section was done on seven species of animals: five mammalian, one amphibian and one avian.

To demonstrate the location of the capillaries, the vascular beds of several muscles were perfused with an opaque, silicone elastomer of low viscosity (Microfil: Canton Biomedical Products, Inc.). The physical properties of this material are given in Appendix III.

As the range in animal size was considerable (frog: 50 grams, - dog: 15 kilograms), the protocol preceding perfusion of the vascular bed had to be modified for each species. The individual protocols used in these experiments are presented in Table XI.

Following intraperitoneal injection of anaesthetic, and intravascular injection of heparin (500 I.U./kg), selected vessels were cannulated for perfusion of the target muscles (Table XI).

In some dogs and cats, a cannula was placed in the popliteal artery for perfusion of the muscles of the calf and lower foot, while in others, the muscles of the head region were perfused by cannulation of both carotid arteries. Perfusion of the muscles of the thigh and lower leg of rabbits was carried out by placing a cannula in the femoral artery. The lower extremities of rats, guinea pigs and frogs

Table XI

Individual experimental protocols employed in the transverse section studies

Animal	No.	Body weight (kg)	Anaesthetic & dose (mg/kg)	Heparin (IU/kg)	Site of cannulation *
dog	7	12-15	pentobarbital (30)	500	popliteal & carotid arteries
cat	12	2.5-4	pentobarbital (30)	500	popliteal & carotid arteries
rabbit	6	2-3	urethane (1500)	500	femoral artery
guinea pig	8	0.5-0.6	pentobarbital (30)	500	abdominal aorta
rat	6	0.2-0.3	pentobarbital (30)	500	abdominal aorta
Japanese quail	2	≈ 0.6	ether	-	-
frog	5	0.05-0.06	urethane (3000)	-	abdominal aorta

* in all experiments involving perfusion, the venous return from the target muscle(s) was also cannulated.

were perfused via cannulation of the abdominal aorta. The muscles of the Japanese quail were not perfused.

The silicone elastomer, which had an initial viscosity of 20 cP (approx.) was injected from a syringe manually. In these experiments the perfusion pressure was not monitored, but in recent experiments of a similar nature it has been found to be in excess of one atmosphere (Willner, personal communication). To ensure proper filling of the vascular bed, the venous outflow was clamped periodically during perfusion, as suggested by Krogh (1919a). The perfusion was maintained for five to ten minutes, at which time only Microfil was observed in the venous outflow. The preparation was then left untouched for 45 minutes to allow the Microfil to set. The muscle(s) to be studied were then removed and placed in 10% formalin buffered at pH 7.4 (Appendix IV).

The muscles used in this study were taken either from the hindlimb (gastrocnemius, soleus, and in some cases, gracilis or tibialis anterior) or from the head (masseter and tongue).

To ensure a high level of perfusion with Microfil, six muscles from three cats (three gastrocnemii and three solei) were pre-perfused with a powerful vasodilator, papaverine, (papaverine hydrochloride, C. E. Frosst Co., Ltd.), 20 mg/100 ml of blood perfused. These muscles were processed and analyzed in the same manner as explained above.

As a further check on the adequacy of capillary perfusion with Microfil, the capillaries of several muscles were identified by staining the mucopolysaccharides in the capillary walls by the

periodic acid-Schiff reaction of Hort (1955) as modified by Hecht (1958). In addition to the modifications of Hecht, care was taken to avoid the use of haematoxylin in the staining procedure. These preparations were analyzed in a manner similar to that employed for the perfusion studies.

After fixation of the whole muscle for 48 hours at 4°C, small pieces of tissue were excised, dehydrated in graded solutions of alcohol, and embedded in paraffin. Transverse sections, 15 μm in thickness, were cut and, in the initial stages of the study, stained with haematoxylin and eosin (H & E) (Figure 9a). The difficulty in distinguishing injected capillaries from haematoxylin stained nuclei, and a desire to assess quantitatively the adequacy of perfusion led to the development of a modified Gomori trichrome stain (GTC), which was routinely used in the rest of the study. The procedures employed in staining with this stain are presented in Appendix V. Sections stained in this manner, exhibit the following characteristics (Figure 9b):

muscle fibres	-	blue-green
collagen	-	green
nuclei	-	very pale purple (if visible at all)
erythrocytes	-	brilliant red
Microfil	-	black (opaque)

Figure 9 - Transverse sections (15 μm thick) from cat gastrocnemius muscle, perfused with Microfil and stained with either haematoxylin and eosin (A) or a modified Gomori trichrome stain (B). In A, the nuclei, located around the periphery of each muscle fibre, are densely stained and thus are easily confused with the injected capillaries. In B, the nuclei are very faintly stained and thus present no problem in the identification of injected capillaries.

Legend:

mf - muscle fibre

c - capillary

n - nucleus

ec - empty capillary

rc - capillary containing a red cell

Scale: 1 cm ≡ 13 μm

Capillaries were counted by direct viewing under the microscope (200x total magnification). In each preparation, 500 to 600 muscle fibres, representing several entire muscle bundles, were examined and the number of capillaries found around the periphery of each fibre (N_{VF}) was recorded. In addition, the capillary and fibre densities were determined from the capillary and fibre counts in five fields of view (area 0.636 mm^2), and using these densities, the C/F ratio was calculated. Finally, the sharing factor (SF) was computed as the quotient $(N_{VF})/(C/F\ ratio)$. Mean values were then determined for each preparation from these individual measurements.

II.2 Longitudinal section studies

Frogs (Rana pipiens) weighing 50 grams (approx.) were anaesthetized by injection of urethane (three to four mg/gram) into the dorsal lymph sac (Gentry and Johnson, 1972). The abdominal aorta was cannulated, and the blood in the hind limbs was washed out by infusing amphibian Ringer solution containing papaverine (Frosst), at a concentration of 0.3 mg/ml, to induce maximal vasodilation. Microfil was immediately infused until it alone appeared in the venous outflow. One hour later, the hind-quarters were removed and fixed for several days in 7% buffered formalin at 4°C. Following fixation, each sartorius muscle was carefully dissected and placed between two glass microscope slides to keep it flat.

Large composites of each muscle were prepared from several consecutive photomicrographs (Figure 10). To determine which of the major vessels were actually arteries, additional sartorius muscles

were infused with higher viscosity Microfil (initial viscosity 450 cP) while being observed under a dissecting microscope. Infusion was stopped when the Microfil just reached the arterial ends of the capillaries. Comparison of these latter muscles with those fully perfused enabled the veins to be easily distinguished from the arteries (Figures 10 and 11).

Personal observations, by vital microscopy, of red cell movements between artery and vein have shown that flow in this muscle is mainly concurrent. This observation, along with a knowledge of the general direction of flow, gained during the injection of the arterial vascular tree alone, made it possible to distinguish 'converging' from 'diverging' pathways at any point in the capillary network.

For ease of measurement, magnified composites of several selected areas were prepared for analysis. One such area, outlined by a black rectangle in Figure 10, is presented in Figure 12. In the left-hand side of this area, the vessels of the arterial inflow (A) may be seen and, a few millimetres to the right, the venules (V) to which the capillaries from A run. It is possible, therefore, to follow along and measure a series of capillary pathways between A and V. A very striking feature is the way in which the capillaries anastomose frequently, each flow path being divided into a number of connected 'segments'. Each segment in the network was assigned an identification number (Figure 12), and each pathway between A and V was characterized by the particular segments making up the pathway. From measurements of the length of each segment, the total length of each possible capillary path was then determined.

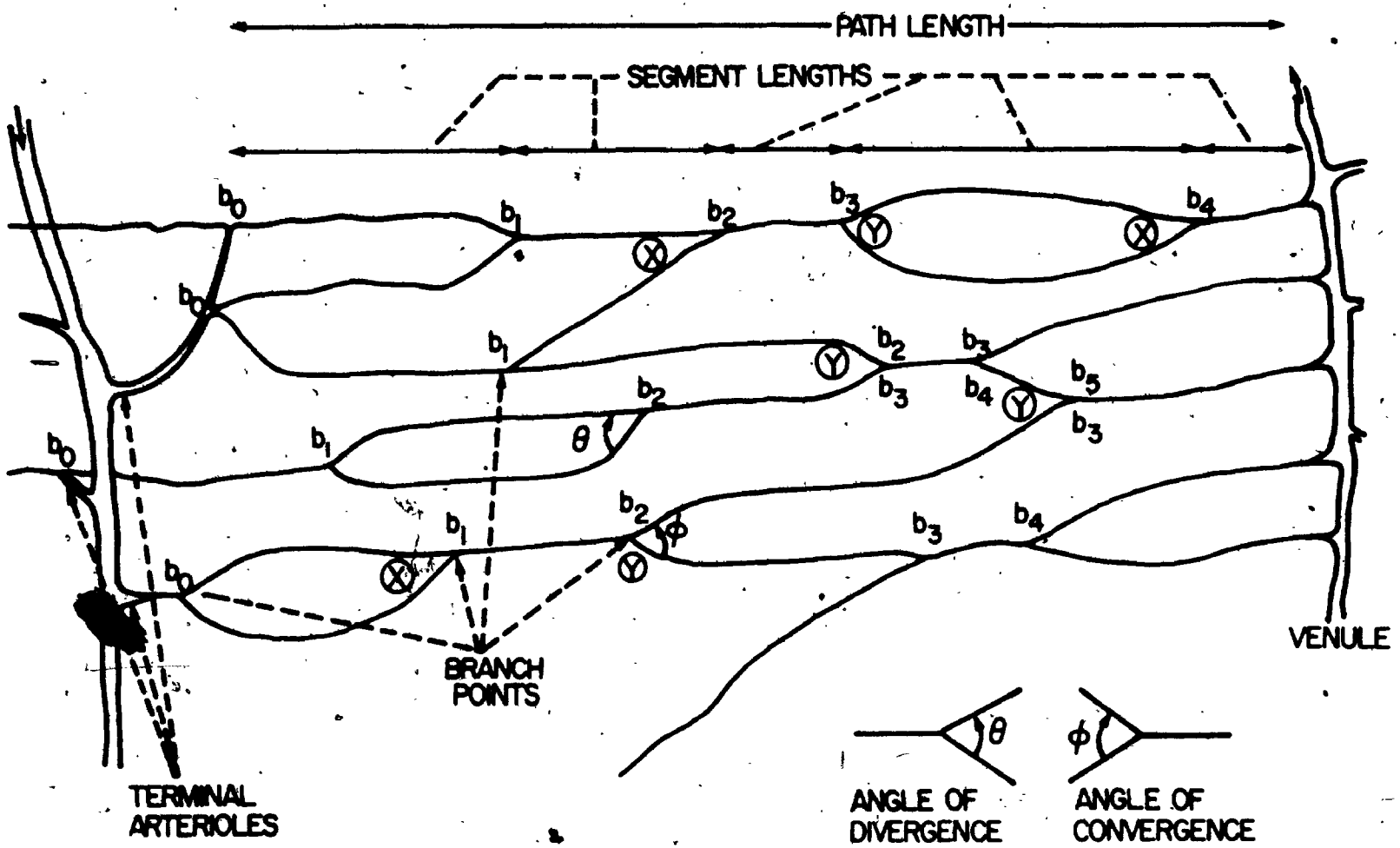

PATH LENGTH
SEGMENT LENGTHS
b_0
b_1
b_2
b_3
b_4
b_5
θ
φ
TERMINAL ARTERIOLES
BRANCH POINTS
VENULE
ANGLE OF DIVERGENCE
ANGLE OF CONVERGENCE
⊗ = REJOINED CAPILLARY
Ⓨ = NEW CAPILLARY

Figure 10 - Composite photomicrograph showing the undersurface of a frog sartorius muscle. The complete vascular system has been outlined by perfusion with Microfil. X and Y denote the proximal and distal ends, respectively. Enlarged composites of areas such as that enclosed by the black rectangle (Figure 12) were used in the analysis of the capillary branching pattern.


5 mm

Figure 11 - Composite photomicrograph showing the undersurface of a frog sartorius muscle in which only the arterial vascular system has been perfused with Microfil. Comparison between partially and fully perfused muscles enabled arterial inputs to be differentiated from venous outflows.

Scale: 1 cm ≡ 1.2 mm

Figure 12 - An enlarged composite photomicrograph of the area within the rectangle shown in Figure 10. Each "segment" of the capillary network has been assigned an identification number to aid in tracing all possible pathways from the arterioles (A) to the venules (V).

1 mm.

Each capillary bifurcation, ie. point of anastomosis to another capillary, was termed a branch point; the point of origin of the capillaries from a terminal arteriole was designated b_o, and subsequent branch points b_1, b_2, ..., b_n, respectively (Figure 13).

The number of capillaries with which a given capillary anastomosed at each branch point was recorded and, to describe the extent of branching, was subdivided as follows:

(i) reference capillary: each flow path in turn was used as the given reference vessel.

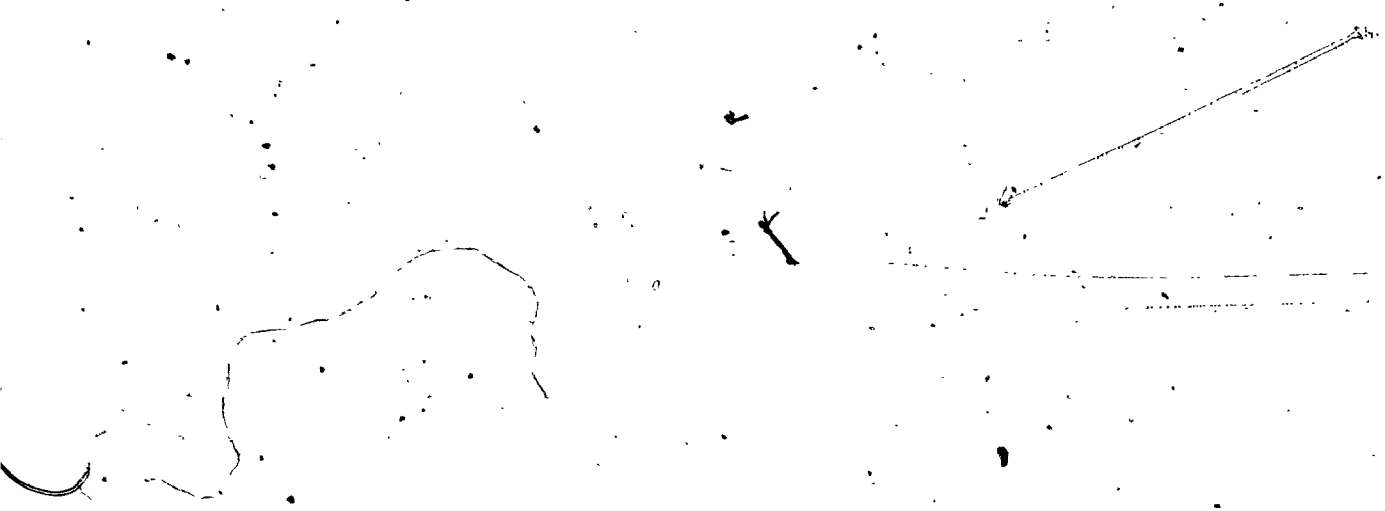
(ii) new capillaries: those capillaries being joined to the reference vessel for the first time.

(iii) rejoined capillaries: those capillaries which had been already connected to the reference vessel at some previous branch point (Figure 14).

In addition the mean angles of convergence and divergence were determined by measurement of the included angle (Figure 14) of 166 capillary junctions.

The depth of focus for the fields of view in the magnified composites (Figure 12) permitted only those capillaries associated with the uppermost layer of muscle fibres to be visualized. Therefore, analysis is restricted to this single layer only. When connections occurred to deeper capillaries, the branch points were determined by referring to the original muscle preparation, focussing up and down as necessary. However, the lengths and points of re-connection of such segments to the network below could not be measured.

Figure 13 - Schematic representation of the capillary network defining terminology and illustrating the "segmental" nature of the network. Beginning at the terminal arteriole (defined as b_0), each point of branching, down the length of the capillary, is numbered consecutively.. The portion of capillary between any two points of branching is termed a segment. The many connections between capillaries provide several alternate routes for blood to pass from arteriole to venule.



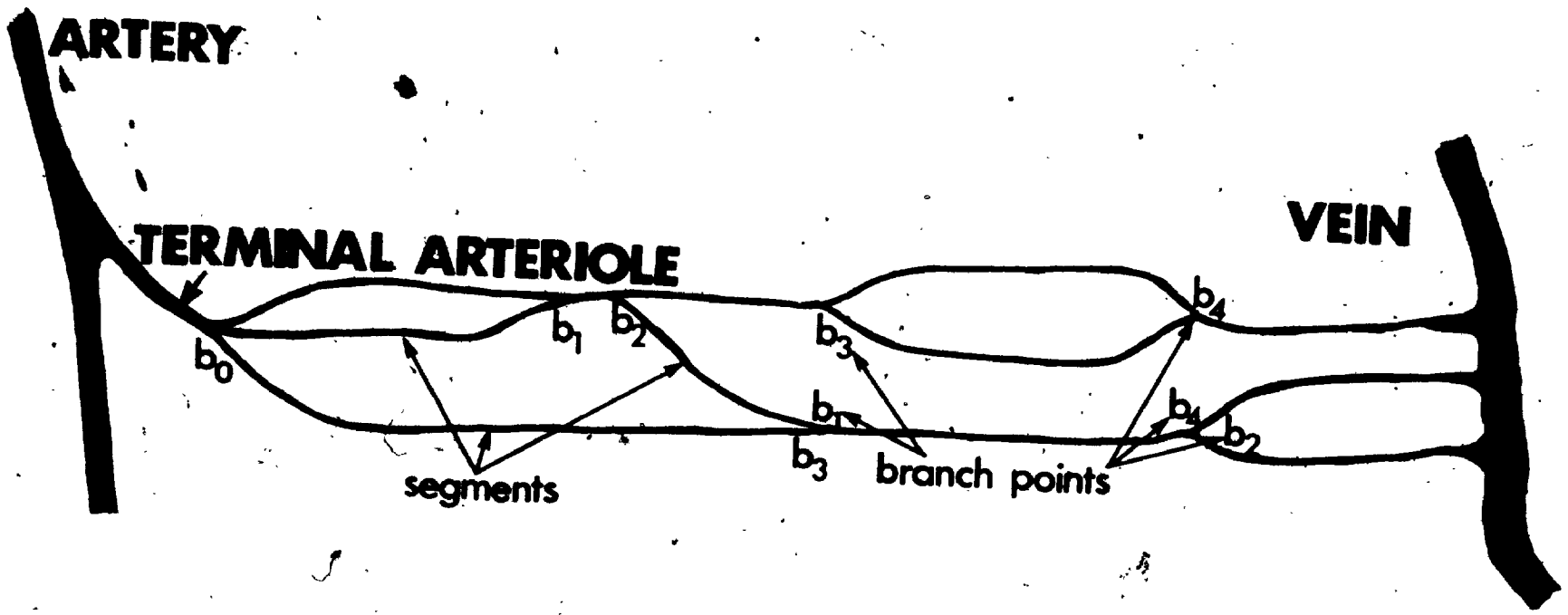

ARTERY
TERMINAL ARTERIOLE
VEIN
b_0
b_1
b_2
b_3
b_4
b_1
b_3
b_4
b_2
segments
branch points

Figure 14 – Schematic representation of the capillary network showing the parameters measured, and defining the terminology used in quantitatively describing the branching pattern.

(i) segment length - the measured distance between any two points of anastomosis.

(ii) path length - the arithmetic sum of the lengths of those segments of which the the path is composed.

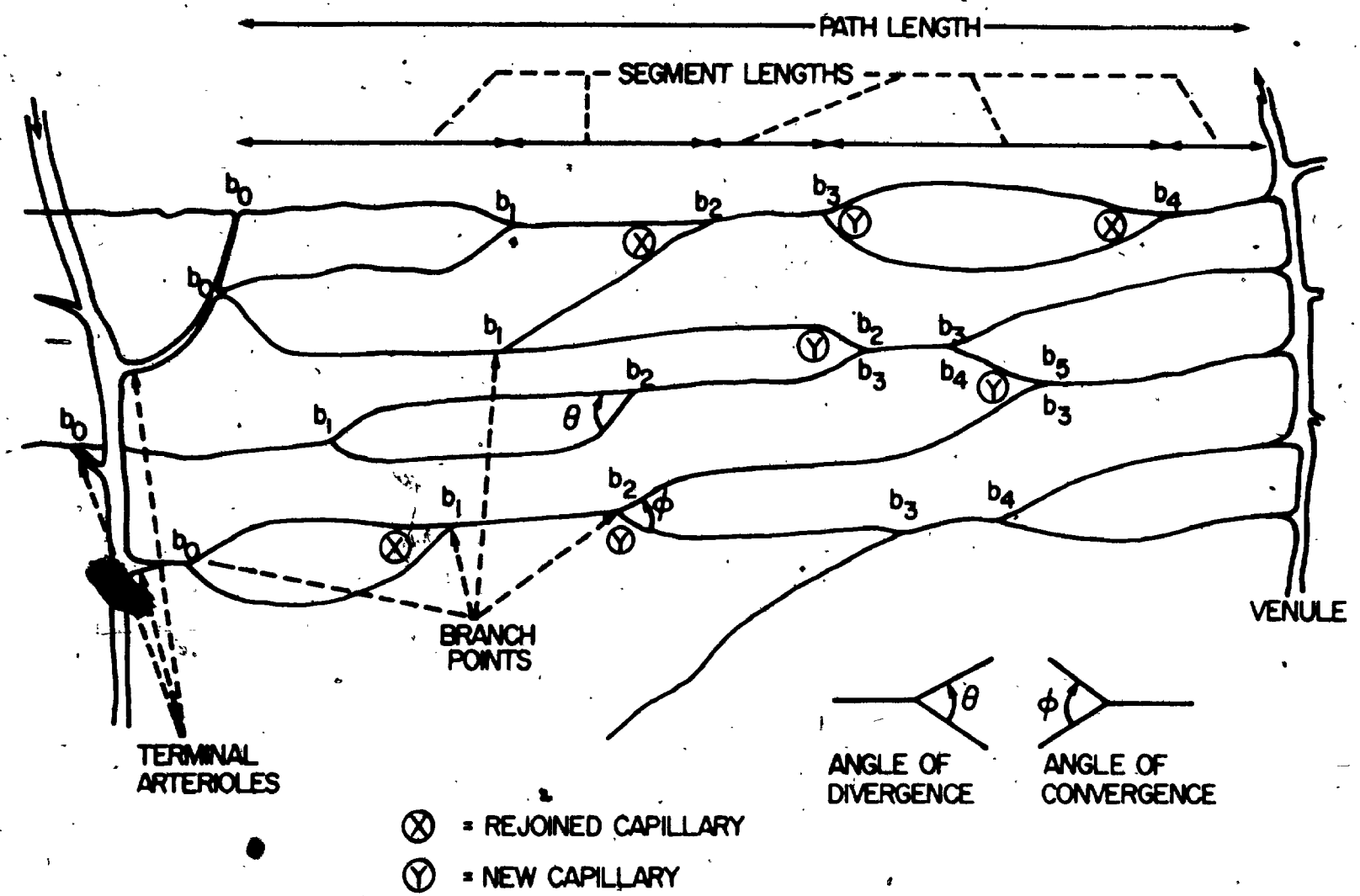
(iii) each distinct pathway from arteriole to venule was taken, in turn, as the reference pathway for the whole network, and the connections between this pathway and all the others in the network defined in terms of their past interaction with the reference capillary:

(a) if the pathway being analyzed had been connected to the reference vessel at any previous branch point, it was termed a "rejoined" capillary.

(b) if the anastomosis joined the pathway being analyzed to the reference capillary for the first time, the pathway was termed a "new" capillary.

(iv) as the general direction of flow was known, each branching point was defined as either converging or diverging, and the included angle between the branches was measured for both types of junctions.

Note: One must be careful not to associate only rejoined capillaries with convergence or new capillaries with divergence; as can be seen in the schematic, both rejoined and new capillaries can result from either convergent or divergent branching.


PATH LENGTH
SEGMENT LENGTHS
b_0
b_1
b_2
b_3
b_4
b_5
θ
φ
VENULE
BRANCH POINTS
TERMINAL ARTERIOLES
ANGLE OF DIVERGENCE
ANGLE OF CONVERGENCE
⊗ = REJOINED CAPILLARY
Ⓨ = NEW CAPILLARY

II.3 Discussion of methods employed

II.3.1 Perfusion technique

Two important considerations when perfusing a microvascular bed are: (1) the physical properties of the casting material, and (2) the need to "override" all local control of the distribution of flow (Sobin and Rosenquist, 1973).

Silicone elastomers (here, Microfil MV-112) have been used extensively to study the microcirculation in various tissues (Sobin and Tremer, 1966). Its physico-chemical properties in the liquid state (low surface tension, viscosity 20-25 cP, uniform particle size < 1 μm, inertness, opacity, etc.: Sobin, Frasher and Tremer, 1962; Sobin, 1966) make it particularly suitable as a microvascular injection medium. When injected following perfusion with a potent vasodilator, complete filling of the capillary bed occurs (Demis and Brim, 1965; Plyley and Groom, 1975). The material undergoes no significant volume change either during polymerization (Sobin et al, 1962; Bassingthwaighte, et al, 1974) or during fixation and storage in aqueous solutions (Sobin, 1966). However, care must be taken when employing hydrocarbon solvents for histological preparations, such as clearing procedures, in that volume changes may result (Sobin, 1966; Bassingthwaighte et al, 1974).

Of course, under-filling of the vascular bed would give falsely low values for the capillary supply. To achieve a high degree of perfusion, a high perfusion pressure was used to inject the relatively low viscosity perfusate. Frequently this procedure was successful, insofar as the major part of the muscle was concerned;

nevertheless, there were often poorly filled areas as well. In this regard, the experience of Krogh (1919a) and Martin et al (1932) is of great interest. Because of poor filling in his experiments, Krogh tried several modifications to improve perfusion. These include: prior infusion of nitrites in warm saline to produce vasodilatation, or tetanizing of the muscles before and during the injection. He concluded, however, that the best results were obtained by the use of intra-arterial injections employing high pressure. Whenever practicable, he tied the vein and set up a very considerable back-pressure before tying the artery. Such procedures were followed in the present study.

Martin et al (1932) found that complete filling of the vascular bed was most difficult, stating that "A striking observation, and one that seems to us probably significant, is that in all these muscles, fasciculi that showed no injected capillaries, or at most a very few, were interspersed among well-injected fasciculi.... Capillary counts were made in injected areas, and in six of the eight muscles under discussion these were very consistent...." Heeding this advice, the study of transverse sections was not conducted on random fields of view, but on well-injected bundles of fibres (fasciculi).

II.3.2 Histological preparation

Initially, the histological sections used for analysis were cut at 10 μm thickness. However, we sometimes observed empty capillaries from which either a red cell, or else a small piece of Microfil, had been dislodged during some phase of the histological

procedures. Occasionally such displaced particles were to be seen superimposed on an adjacent muscle fibre. In the present experiments, only 15 μm thick histological sections were used, and under these conditions this problem has not been encountered.

The histological sections were stained with either haematoxylin and eosin (Figure 9a) or a modified version of Gomori's trichrome stain (Figure 9b). Sections stained with H & E proved most difficult to analyze, for, at magnifications sufficiently low to analyze whole muscle bundles, it was most difficult to distinguish nuclei (both are located around the periphery of a transversely-sectioned muscle cell: Figure 9a). To facilitate identification of capillaries, the sections were stained with the modified Gomori trichrome stain (Plyley and Groom, 1975). One of the modifications made to the staining procedure was the elimination of any nuclear stain; this results in the nuclei appearing very faint purple in colour (if they are visible at all: Figure 9b), and presenting no problem to capillary counting. A second benefit of using this staining procedure was that any red cells, remaining in those capillaries which had remained unperfused with Microfil, appeared a brilliant scarlet (Figure 9b). Such staining has made possible a quantitative assessment of the success of capillary perfusion with Microfil. For any muscle bundle, the relative number of capillaries containing red cells and Microfil were compared; in those bundles used for analysis, the median value of the percentage of capillaries containing Microfil was 87% (range 50% - 99%). In all counting procedures, the number of capillaries containing red cells was included in the counts. However, there is the possibility

that other capillaries, also unperfused with Microfil, may have been sectioned through regions of cell-free plasma. In well-filled areas this would be unimportant, but in the poorly-filled sections, a significant underestimate of the actual capillary count might result. This possibility was tested in two ways: (1) pre-infusion of papaverine to promote maximal vasodilatation and (2) staining histochemically for a component in the capillary wall (mucopolysaccharide) by the periodic acid-Schiff (PAS) reaction. However, owing to difficulties in making the PAS method consistent, only limited use of this histochemical procedure was possible†.

The shrinkage which occurs during the processing of skeletal muscle for light microscopy amounts to 50% (approx.) in area (Heroux and St. Pierre, 1957; Goldspink, 1961; Moore et al, 1971). This suggests that most reported values of capillary and fibre densities (including those presented here), measured from sections of tissue fixed in formalin and embedded in paraffin, should be multiplied by a factor of 0.50 (approx.). However, dimensionless parameters which relate capillaries to fibres rather than to area, such as the number of capillaries around a fibre, the capillary to fibre ratio and the sharing factor, are independent of tissue shrinkage, making no correction necessary.

† An improved method for the PAS reaction was developed in 1976, and has recently been submitted for publication (Appendix VI).

II.3.3 Analysis via direct viewing

The analysis of the transverse sections of muscle was made on histological sections viewed directly through the microscope. This procedure has two disadvantages compared with analyzing composite photomicrographs: (1) there is no record for later use and (2) subjective decisions on the part of the observer lead to less accurate measurements. On the other hand, the large number of measurements made here would improve the accuracy of the method employed, and it is doubtful that the extra time, materials, etc. required to produce the composites would yield a substantially increased accuracy. However, a permanent record for later reference would be quite valuable for further analysis or discussion.

II.3.4 Sartorius muscle preparation

The sartorius muscle of frog is a thin, flat muscle (dimensions - 35 x 7 x 0.7 mm approx.), and the organization of the blood vessels may well differ from that found in thicker muscles, in this or other species. However, the vascular architecture seen in the composite photomicrographs corresponds quite closely to that described by Hammersen (1962, 1968, 1970) for various muscles in the hindlimbs of cat and rabbit.

In the present study, only those vessels nearest the undersurface of the muscle were analyzed, and it is possible that significant differences from the observed pattern could exist in deeper layers of the muscle. However, the analysis presented here is applicable to other patterns of capillary branching.

The composite photomicrographs (Figures 10-12) are two-dimensional projections of a three-dimensional structure, and therefore, any segments of capillaries not running parallel to the plane of section, ie. joining a capillary deeper in the section, will appear foreshortened. Similarly, the measurements of the angles of convergence and divergence must be minimum values. However, the error incurred in both the segment and path length measurements will be small, as the mean path length is more than 50 times the mean fibre diameter.

Shrinkage of tissue is not a problem in this preparation as no histological processing, except for fixation in 7% buffered formalin, is necessary. Hort (1971) reports that heart muscle treated in this way incurs a shrinkage of only one to two percent.

"How often have I said to you that when you eliminated the impossible, whatever remains, however improbable, must be the truth?"

(Sir Arthur Conan Doyle)

III. DIRECT VISUAL ANALYSIS OF TRANSVERSE SECTIONS OF STRIATED MUSCLE

III.1 Results

III.1.1 Verification of capillary filling

The completeness of filling the vascular bed with Microfil was assessed by counting the capillaries containing red cells in each section. Usually many well-filled bundles were observed in each section and these were used for analysis. On several occasions, however, the degree of vascular filling was poor. Furthermore, it was considered probable that in 15 µm sections, not all capillaries unperfused with Microfil would contain red cells; indeed, many may have contained only plasma and would therefore remain undetected during counting. For this reason, counting was restricted to sections where the number of capillaries containing red cells was < 10%; capillaries identified in this way were included in all vessel counts. However, later comparisons of poorly- and well-filled sections revealed that no significant difference existed between the two (Table XII). As this was a consistent finding in all species examined, those muscles found to be poorly perfused were included in the results presented here.

Table XII

Dependence of capillary counts of N_{VF}, from contralateral muscles, or from muscles of two animals, on the percentage perfusion

Animal, muscle, % perfusion	Percentage of fibres surrounded by 0-9 vessels 0	1	2	3	4	5	6	7	8	9	Mean value of frequency distribution ± SE (n)
Rat 1, L.gastroc 70%	0.9	5.3	16.9	23.3	23.4	20.1	7.4	1.7	0.7	0.4	3.86 ± .04 (543)
Rat 1, R.gastroc 98%	0.9	4.2	13.6	23.9	25.9	17.9	10.1	2.2	1.3	-	3.84 ± .06 (552)
Rat 2, L.gastroc 98%	0.2	2.6	11.8	22.7	28.4	20.4	9.4	4.1	0.4	0.2	3.81 ± .08 (543)
Cat 1, masseter 91%	0.2	4.1	21.6	41.5	26.6	5.3	0.8	-	-	-	3.09 ± .04 (533)
Cat 2, masseter 57%	-	2.7	14.7	42.1	33.1	7.0	0.4	-	-	-	3.28 ± .02 (511)

III.1.2 Verification of counting procedures

The reproducibility of the counting procedures was checked by comparing results obtained from adjacent sections of a muscle counted one day apart. In this way, different bundles would undoubtedly be examined. An example of this, taken from masseter of cat, is presented in Table XIII, and shows excellent agreement between the two sets of data ($p > .80$).

III.1.3 Relationships of capillaries and muscle fibres in 19 striated muscles

In all 19 muscles examined, the number of capillaries around a fibre, N_{VAF}, lay between 0 and 9 (Table XIV). Moreover, the percentage of fibres surrounded by a particular number of capillaries was remarkably constant from one muscle to another as can be seen by examining any vertical column. As a result of this consistent finding, the overall mean percentages were calculated and appear at the bottom of each column ($\pm$ SE).

The mean value for N_{VF} ranged from 2.92 (in frog sartorius) to 4.01 (in rat soleus), each mean value being calculated as the grand mean of the mean values obtained from each of the "n" muscle preparations examined. These grand mean values ($\pm$ SE) are presented in the last column of Table XIV. (NOTE: The standard error given is not the standard error of the mean of the distribution of each row, but is the result of averaging the mean values of the distributions obtained from "n" experiments, e.g. for gastrocnemius of cat, the mean value of $3.76 \pm .13$ (SE) is the result of averaging the mean values:

Table XIII

Capillary counts made on two serial sections counted one day apart

Cat masseter % perfusion	Percentage of fibres surrounded by 0 - 9 vessels										Mean value of frequency distribution ± SE. (n)
	0	1	2	3	4	5	6	7	8	9	
85%	0.2	5.0	21.5	39.6	26.9	6.1	0.8	-	-	-	3.10 ± .04 (606)
91%	0.2	4.1	21.6	41.5	26.6	5.3	0.8	-	-	-	3.09 ± .04 (533)

Table XIV

Numbers of capillaries found around the periphery of muscle fibres (N_{VF}) from seven species of animal

Animal, muscle	Percentage of fibres surrounded by 0 - 9 vessels										Mean value ± SE for "n" experiments	
	0	1	2	3	4	5	6	7	8	9		
Cat												
gastroc.	0.7	3.0	11.5	24.5	33.9	21.6	5.4	1.1	-	-	3.76 ± .13	(5)
soleus	0.6	4.3	12.5	21.9	33.1	19.1	6.6	1.7	0.2	-	3.80 ± .14	(5)
masseter	0.1	3.8	18.9	38.9	30.3	7.3	0.7	0.1	-	-	3.21 ± .06	(3)
tongue	0.2	5.1	17.5	40.0	25.1	10.2	1.5	0.2	0.2	-	3.23	(1)
Dog												
gastroc.	0.2	2.7	12.7	29.9	32.7	16.6	4.0	1.2	0.1	-	3.65 ± .13	(4)
soleus	-	1.1	8.6	26.3	35.0	22.3	5.6	1.0	0.1	-	3.90 ± .07	(3)
masseter	0.2	2.6	12.9	30.7	32.0	17.0	4.0	0.8	-	-	3.60	(2)
tongue	0.2	6.2	18.9	32.2	25.4	14.5	2.0	0.6	-	-	3.22	(1)
Rat												
gastroc.	0.7	4.0	14.1	23.3	25.9	19.5	9.0	2.7	0.8	0.2	3.84 ± .02	(4)
soleus	0.4	2.9	13.0	20.2	28.5	20.3	9.0	4.1	1.3	0.2	4.01 ± .20	(3)
Guinea pig												
gastroc.	0.8	6.6	17.7	30.3	28.3	12.4	2.9	0.7	0.1	-	3.34 ± .06	(7)
soleus	0.5	6.0	19.8	32.0	27.4	11.4	2.1	0.6	-	-	3.26 ± .05	(4)
tibial. ant.	0.9	4.5	18.3	33.5	28.1	11.6	2.2	0.7	-	-	3.45	(1)
gracilis	0.2	2.9	19.0	30.8	29.3	13.4	3.4	1.0	-	-	3.31	(1)

Rabbit												
gastroc.	0.8	6.8	17.2	28.6	25.3	14.8	5.1	1.1	0.4	-	3.42 ± .08	(6)
soleus	0.6	5.7	16.6	29.9	29.3	13.2	3.9	0.6	0.3	-	3.41 ± .20	(6)
Japanese quail												
gastroc.	0.8	5.3	18.7	35.4	27.3	9.9	2.2	0.3	0.2	-	3.25 ± .05	(2)
Frog												
gastroc.	0.4	6.0	24.3	36.8	24.9	6.5	0.9	0.1	0.1	-	3.03 ± .10	(5)
sartorius	1.5	6.5	25.8	35.2	23.3	5.5	1.8	0.5	0.1	-	2.92 ± .14	(5)
Mean value	0.52	4.53	16.74	30.55	28.69	14.22	3.81	1.01	0.21	0.02	3.45 ± .07	(19)
± SE (n = 19)	.08	.38	.99	1.27	.78	1.23	.56	.22	.08	.01		

3.68, 3.75, 3.38, 4.15, 3.86 obtained in the five separate experiments on this muscle).

For completeness and comparison with past studies, the capillary and fibre densities (N_C/mm^2 and N_F/mm^2 respectively) were determined. The capillary density values ranged from $144/mm^2$ in frog sartorius to $1440/mm^2$ in cat tongue; the fibre densities (ranging from $135/mm^2$ in frog sartorius to $1039/mm^2$ in tongue of cat) gives an indication of the range of fibre sizes encountered.

The capillary/fibre ratio (C/F ratio), calculated from the capillary and fibre densities, ranged from 1.02 (tongue of cat) to 2.05 (rat soleus) with a mean value $1.44 \pm .06$ (SE). The sharing factor (SF) or mean number of fibres sharing one capillary, was computed as the quotient (N_{VF}/(C/F ratio)). These values ranged from 1.99 (rat soleus) to 3.22 (tongue of dog) with a mean value of $2.48 \pm .07$ (SE). The mean values ($\pm$ SE) of N_C/mm^2, is N_F/mm^2, C/F ratio and SF for the 19 muscles examined are presented in Table XV.

Pre-infusion of papaverine provided a much more consistent filling of the capillaries with Microfil, as < 2% of the capillaries were found to contain red cells. The frequency distributions for N_{VF} from these preparations (cat gastrocnemius and soleus) are presented in Table XVI. The increase in mean N_{VF} in these muscles was found to be + 0.01 in gastrocnemius and +0.12 in soleus; neither increase was found to be statistically significant ($p > .80$).

Due to the "patchy" nature of the PAS reaction, it was impossible to analyze whole muscle bundles. However, analysis of three gastrocnemii and solei yielded results which differed slightly

Table XV

Additional parameters used in describing the relationship of capillaries to muscle fibres

Animal, muscle	Capillary density $(mm^2)^{-1}$	Fibre density $(mm^2)^{-1}$	Cap/fibre ratio	Sharing factor
Cat				
gastroc.	369	221	1.62	2.26
soleus	435	247	1.76	2.43
masseter	621	552	1.13	2.85
tongue	1440	1039	1.39	2.33
Dog				
gastroc.	706	499	1.45	2.58
soleus	719	477	1.55	2.59
masseter	820	693	1.20	3.01
tongue	1008	987	1.02	3.22
Guinea pig				
gastroc.	677	498	1.41	2.39
soleus	725	577	1.27	2.58
tibial. ant.	1210	876	1.38	2.50
gracilis	853	583	1.46	2.26
Rat				
gastroc.	487	257	1.87	2.08
soleus	396	195	2.05	1.99
Rabbit				
gastroc.	341	209	1.67	2.07
soleus	371	245	1.67	2.21
Japanese quail				
gastroc.	349	263	1.32	2.47
Frog				
gastroc.	212	184	1.15	2.65
sartorius	144	135	1.05	2.71
Mean value ± SE (n=19)	n.a.	n.a.	1.44 .06	2.48 .07

from the Microfil studies (Table XVI). In each preparation, the percentage of fibres surrounded by either zero or one capillary was decreased and the percentage surrounded by four capillaries was increased slightly, resulting in a somewhat skewed distribution. The mean values of N_{VF} were increased by 0.20 in gastrocnemius and 0.18 in soleus, but were not significantly different ($p > .30$) from the mean values obtained in the perfusion studies.

Data for N_C/mm^2, N_F/mm^2, C/F ratio and SF for the papaverine pre-infusion and PAS experiments are presented in Table XVII. While the values for the C/F ratio and SF are not significantly different from those found in the perfusion studies, the capillary and fibre densities from the PAS experiments were found to differ significantly. This result was due to greater shrinkage during the histological processing for the PAS reaction.

III.2 Discussion

III.2.1 Methods

Most early investigators routinely injected a solution of gelatin and India ink (or dye) into the capillaries. After standard histological processing, the sections were quite often stained using procedures involving the use of haematoxylin. However, our experience would suggest caution in accepting results obtained from sections stained with haematoxylin (section I.7). The particulate nature of the perfusate, the common location of both cell nuclei and capillaries around the fibre periphery, and the dark appearance of the

Table XVI

Numbers of capillaries found around the periphery of muscle fibres (N_{VF}) in cat gastrocnemius and soleus determined from three experimental preparations

Muscle, exper. preparation	Percentage of fibres surrounded by 0-9 vessels										Mean value ± SE for "n" experiments
	0	1	2	3	4	5	6	7	8	9	
Gastroc. non-vasodilated	0.7	3.0	11.5	24.5	33.9	24.6	5.4	1.1	-	-	3.76 ± .13 (5)
vasodilated	0.2	1.3	7.6	28.9	33.7	19.4	5.1	1.4	0.2	-	3.77 ± .11 (3)
PAS	0.2	1.8	7.3	27.9	29.8	23.5	7.1	2.1	0.8	-	3.96 ± .10 (3)
Soleus non-vasodilated	0.6	4.3	12.5	21.9	33.1	19.1	6.6	1.7	0.2	-	3.80 ± .14 (5)
vasodilated	-	0.9	5.6	28.1	38.5	21.1	4.1	1.5	0.2	-	3.92 ± .16 (3)
PAS	-	0.7	5.2	26.8	36.9	22.6	6.3	1.1	0.4	-	3.98 ± .12 (3)

Table XVII

Additional parameters used in describing the relationship of capillaries to muscle fibres as determined from three experimental preparations

Muscle, exper. preparation	Capillary density $(mm^2)^{-1}$	Fibre density $(mm^2)^{-1}$	Cap/fibre ratio	Sharing factor
Gastroc.				
non-vasodilated	369	221	1.62	2.26
vasodilated	397	248	1.60	2.32
PAS	899	544	1.65	2.42.
Soleus				
non-vasodilated	435	247	1.76	2.43
vasodilated	460	277	1.66	2.34
PAS	617	347	1.78	2.34

haematoxylin-stained nuclei makes counting of capillaries most difficult unless viewed at higher magnifications. However, the use of higher magnifications for analysis makes it very difficult to analyze complete muscle bundles which we feel is necessary when looking at the geometric relation of capillaries and muscle fibres.

Any under-filling of the capillary bed, would lead to falsely low values for all parameters describing the capillary-muscle fibre relationship. In the past, it has been impossible to assess quantitatively the degree of vascular filling. In the present study, this has been achieved by staining the sections with the modified GTC stain, thereby allowing identification of any capillaries, unperfused by Microfil but still containing red cells, by the intense red staining of the erythrocytes within them.

III.2.2 Comparison of Microfil technique with the PAS technique

Pre-infusion of the vascular bed with papaverine produced a much more uniform perfusion of the capillaries but only marginally increased the observed mean values for all descriptive indices. Similarly, results obtained using the histochemical stain PAS were slightly greater but not significantly different (Tables XVI and XVII). These results can only be interpreted to mean that, under the conditions of these experiments, the number of capillaries, which went undetected by chance sectioning through regions of plasma, must be quite small.

III.2.3 The capillary supply of skeletal muscle fibres

Several methods have been used to demonstrate the vascular beds in skeletal muscle, and the capillary supply of skeletal muscles has been described in many ways. These have been critically discussed in sections I.6 and I.7 respectively. Therefore any discussion at this point, concerning a particular method or descriptive index will be brief and reference will be made instead to these sections for a more detailed critique. Capillary density, although widely criticized (Pappenheimer et al, 1951; Pappenheimer, 1953; Hammersen, 1970; Plyley and Groom, 1975), was measured in this study and the results presented for completeness only. The C/F ratio was shown earlier to be highly dependent on both the method of measurement and the type of vascular arrangement being studied (section I.7). However, any discussion on modelling the vascular bed in relation to the muscle fibres would be impossible without some idea of its value (and both N_{VF} and SF as well).

The agreement between the different muscles, regarding the frequency distribution for the number of capillaries surrounding a fibre (N_{VF}) is quite remarkable. Moreover, these muscles, taken from seven different species and coming from widely different anatomical sites (hindlimb, jaw, tongue), represent a 23-fold difference in cross-sectional fibre area! This suggests that the mean percentages listed at the foot of Table XIV may well be representative of a wider range of striated muscles.

Although the values of capillary density ranged from 144/mm^2 to 1440/mm^2 (Table XV), the frequency distribution for N_{VF} was nearly the same in all cases. This makes it clear that any differences in

capillary density are primarily a consequence of differences in mean fibre size. It appears reasonable to hypothesize that differences in mean fibre size are indicative of different metabolic rates for each fibre, and therefore, in the required rate of oxygen supply. It would seem that Nature prefers to increase oxygen supply by decreasing the mean diameter of muscle fibres rather than by increasing the number of primary sources of oxygen around the muscle fibre.

The observed range of values for N_{VF}, zero to nine, is in complete agreement with the reported observations for the capillary supply of skeletal muscle fibres in several species (Table XVIII). Although these studies report the capillary supply for the various types of fibres in the muscles studied (for classification of fibre type see section I.3), the overall range of reported values for muscle as a whole does not appear to be different from the observed range in this study.

The mean values for N_{VF} ranged from 2.9 to 4.0 (Table XIV). Several authors (Table XIX) have reported mean values for the number of vessels surrounding each type of fibre. The observed range of values here reported is not in agreement with those of Valdivia and co-workers (1958, 1960). The rather high values reported by these authors in the 1958 paper probably result from the methods employed. Valdivia had hoped to show, via India ink injection, that the vascular supply of skeletal muscle in altitude-adapted animals was greater than that at sea level. However, it seems likely that his use of haematoxylin would lead to confusion between the peripherally-located nuclei and capillaries (section I.7).

Table XVIII

Values of N_{VF}, for the various types of muscle fibres, reported in the literature

Year	Author(s)	Animal(s), muscle(s)	Fibre type A	B	C	Overall range of values
1950	Bösiger	bird breast				0-6
1964	Henneman and Olson	cat gastroc. and soleus	0+	4-8	4-8	0-8
1965	Romanul	rat or rabbit gastroc. and soleus	1-2	4-8	4-8	1-8
1965	Nishiyama	cat or rat gastroc. and soleus	1-2	3-4	4-6	1-6
1973	Taylor et al.	cat masseter				3-4
1974	Asmussen and Kiessling	frog -	2	4-5	4-5	2-5

+ unless the A fibre is surrounded by B and C fibres.

In the second paper (1960), an attempt was made to show that the number of functioning capillaries was greater in the muscles of altitude-stressed animals (8-10 months at an altitude of 16,500 feet). Indeed, it appears that the observed increase is quite significant (an increase from 2.4 to 4.3); however, this result must be questioned. First, the identification of capillaries by staining the erythrocytes within them has been criticized previously (section I.7). Second, one might expect to see an increased vascular supply using this method as a result of a higher haematocrit in the altitude-adapted animals. A higher haematocrit would lead to an increased probability of an erythrocyte being found within a sectioned capillary. The results, however, do not show any increase in the number of capillaries in the white area of the muscle! A possible explanation for these findings would be a marked difference in the reactivity of the vascular beds of the red and white areas of muscle to some unknown, altitude-induced stimulus. Such a difference in vascular reactivity is quite probable, for a number of studies have shown a distinct difference in the responses of the primarily 'white' gastrocnemius and 'red' soleus muscles of cat to induced hyperaemia (Hilton and Vrbová, 1968; Folkow and Halicka, 1968; Reis et al, 1969; Hudlicka, 1969; Hilton et al, 1970; Allun et al, 1974; Forrester and Hamilton, 1975). Variations in capillary sensitivity to reactive hyperaemia (Burton and Johnson, 1972; Gentry and Johnson, 1972) and in capillary control (Johnson and Wayland, 1967; Webb and Myers, 1973; Gray, 1971, 1972, 1973) have been shown previously. Regarding arteriolar response to reactive hyperaemia, Gentry and Johnson (1972) state, "Probably the unresponsiveness...

reflected trauma incurred in the preparation or the depth of anaesthesia. However, non-reactivity may be normal for some vessels, making generalizations difficult."

Both papers by Valdivia and associates (1958, 1960) report an altitude-induced increase in the capillary supply of individual muscle fibres. More recent studies (Banchero, 1975; Eby and Banchero, 1976) dispute these results on the grounds that the methods employed by Valdivia and co-workers do not allow any indication "as to whether the increased number of capillaries was due to the development of new vessels and/or the recruitment of previously closed ones." (Eby and Banchero, 1976). The results of Banchero and his colleague, show that although there is an increased capillary density as a result of altitude adaptation, it is the result of a concomitant decrease in mean fibre size, resulting in no change in the capillary/fibre ratio.

The mean values of N_{VF} reported for the fibres of the gastrocnemius and soleus of guinea pig (Table XIX: Mai et al, 1970) are somewhat greater than the range 3.26-3.34 found in the present study. No satisfactory explanation for the discrepancy has been found as yet; possibly further studies will have to be done before any definite conclusions can be drawn. The mean values for N_{VF} of cat muscle obtained in the present study (range 3.21 to 3.80) are in excellent agreement with the observations of Eriksson and Myrhage (1972); these authors, using histochemical methods to identify capillaries, found 3.5 to 3.8 (overall mean 3.6) capillaries around the various types of fibres in cat tenuissimus (Table XIX). No other reports of N_{VF} have been found.

Table XIX

Mean values of N_{VF}, for the various types of muscle fibres, reported in the literature

Year	Author(s)	Animal(s), muscle(s)	Fibre Type A	B	C	Range of mean values
1958	Valdivia	guinea pig vastus lateralis[+]	red area only			6.4-7.8
1960	Valdivia et al	guinea pig vastus lateralis[+]	red area			2.4-4.3[++]
			white area			1.0
1970	Mai et al	guinea pig gastroc. and soleus[+++]	3.9	4.5	5.3	3.9-5.3
1972	Eriksson and Myrhage	cat tenuissimus	3.5	3.6	3.8	3.5-3.8

[+] vastus lateralis is composed of two distinct areas, one red, the other white

[++] the value 2.4 is for control animals
the value 4.3 is altitude-adapted animals

[+++] four types of fibres were distinguished, but type 4 was included under type B

The mean of the frequency distribution of the number of capillaries around a fibre (Figure 15) was 3.47 ± 1.26 (SD). As the observations of N_{VF} yields discrete values only, the distribution of N_{VF} was expected to follow either the binomial or Poisson probability distributions. However, to follow a Poisson distribution, the mean value must be small relative to the maximum number of possible events per sampling unit. Using a mean fibre diameter of 60 µm, and an average capillary diameter of 3.5 µm, it was found that the maximum number of capillaries, which could be placed side by side around the fibre, was approximately 55. As the ratio of the mean to this maximum value is 0.06, it seems unlikely the probability distribution of N_{VF} is Poisson.

The fit of the observed data to a binomial distribution of probability 0.38 was tested using the chi-squared test for goodness of fit (Table XX). The value 0.38 was determined from the quotient (mean/maximum number of observed events, ie. 3.47/9). The fit to the binomial distribution was found to be rather poor (χ^2 = 4.91; degrees of freedom = 4; p = .30). In light of this finding and the following statements by Sokal and Rohlf (1973), it was decided to test the fit of the distribution of N_{VF} to a normal distribution. Sokal and Rohlf (p. 79) state, "We may fit continuous frequency distributions to some sets of meristic data as, for example, the number of teeth in an organism. In such cases, we have reason to believe that underlying biological variables causing differences in numbers of the structures are really continuous, even though expressed as a discrete variable."

Figure 15 - Frequency distribution of the number of capillaries observed around the perimeter of muscle fibres taken from the head and/or hind limb regions of five mammalian, one amphibian, and one avian species. Each bar represents the mean of 19 determinations (from Table XIV), and the error bars are the standard error of the mean. The distribution is Gaussian ($x^2 = 0.18$; degrees of freedom = 3; $p < .99$), having a mean value of 3.47 ± 1.26 (S.D.; n = 100); it is slightly leptokurtic ($g_1 = 0.52$; df = 99; $p < .04$) and not significantly skewed ($g_2 = 0.30$; df = 99; $p > .50$).

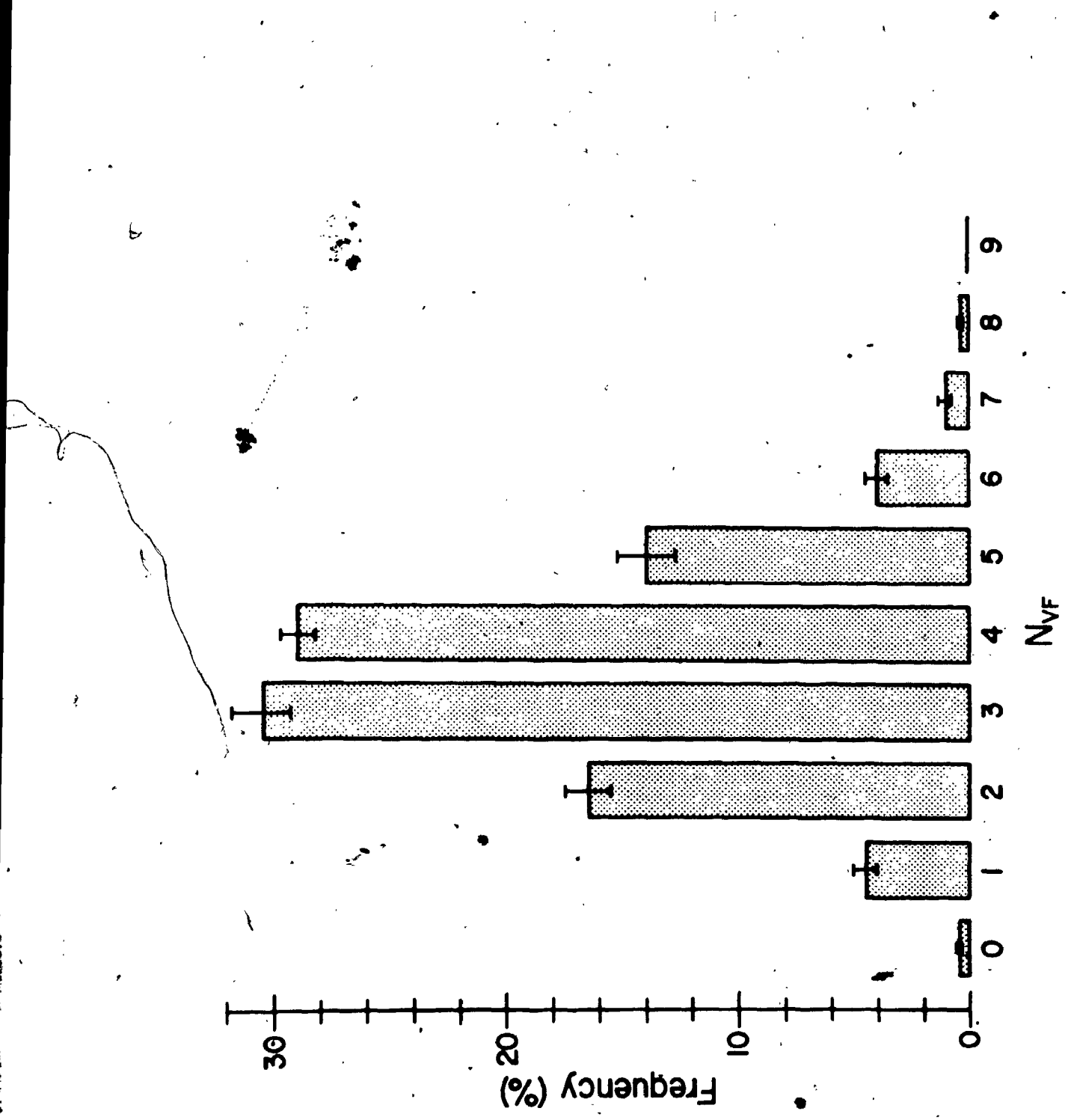

Frequency (%)
0
10
20
30
0
1
2
3
4
5
6
7
8
9
N_{VF}

TABLE XX

Chi-squared tests for the goodness of fit of the experimental data to both the binomial and normal distributions

N_{VF}	Frequency		
	Observed	Expected[+]	Expected[++]
0	0.52	1.43	0.70
1	4.53	7.65	4.56
2	16.74	18.32	16.01
3	30.55	25.91	29.53
4	28.69	23.82	29.06
5	14.22	14.76	15.27
6	3.81	6.15	4.21
7	1.01	1.66	0.61
8	0.21	0.26	0.05
9	0.02	0.02	0
χ^2-value		4.91	0.18
df		4	3
p		> .25	> .99

[+] for a binomial distribution with the probability of the event equal to 0.38 (mean/observed maximum number of events, ie. 3.46/9).

[++] for a normal distribution with the same mean and variance as the observed data.

When the observed data were tested against a normal distribution of the same mean and standard deviation as the observed data (Table XX), the fit was found to be excellent ($\chi^2 = 0.18$; degrees of freedom = 3; $p = .99$).

Although the observed data appear to follow the normal distribution, there exists the possibility that the data actually follow a repulsed binomial distribution. Sokal and Rohlf (p. 62) state that, "Two main types of departure from expectation are likely: (1) clumping and (2) repulsion,.... The clumped frequencies...have an excess of observations at the tails of the frequency distribution and consequently a shortage of observations at the center. Such a distribution is called contagious.... In the repulsed frequency distribution there are more observations than expected at the center of the distribution and fewer at the tails." As there are more observations than expected in the central region of the frequency distribution and fewer than expected in the tails (Table XX), it appears that distribution is repulsed. Concerning repulsed distributions, Sokal and Rohlf (p. 63) point out that the observation of a repulsed distribution "is more difficult to interpret biologically. There are fewer homogeneous groups and more mixed groups in such a distribution. This involves the idea of a compensatory phenomenon...", which is entirely possible on the grounds that while zero or one capillary is inadequate, three or four capillaries may be optimal for the delivery of oxygen to skeletal muscle fibres. The pivotal question concerning the best fit to the observed data is whether the placement of each capillary around a fibre is an endependent event, following the normal distribution, or whether the placement of each capillary has a direct effect on the placement of any subsequent capillary,

corresponding to a repulsed binomial distribution.

The capillary/fibre ratios (C/F ratio) obtained in this study ranged from 1.02 to 2.05 with an overall mean of 1.44 ± .06 (SE; n = 19) (Table XV). Values reported in the literature for the C/F ratio of various muscles from the same seven species range from 0.3 to 4.0 (Appendix II). Even for the same muscle, the results vary considerably, eg. rabbit semitendinosus 1.22 (Stoel, 1925), 3.06 (Duyff and Bouman, 1927); human quadriceps femoris 0.81 (Parizkova et al, 1971), 1.30 (Andersen, 1975).

Great variations in observed values for capillary density are to be expected due to the great variability in muscle fibre size, shrinkage incurred in processing and counting techniques employed (sections I.6 and I.7). However, the substantial differences observed between reported values of C/F ratio were not anticipated. The C/F ratio is dependent on two factors, the number of capillaries around each fibre (N_{VF}) and the sharing factor (SF). One major omission in the present study was that the sharing factor was not measured directly but was computed as the quotient of N_{VF} and the C/F ratio. As N_{VF} is rather constant (Table XIV), the observed differences in calculated sharing factors will reflect mainly the variations in C/F ratio. This is unfortunate for the sharing factor is most important when describing the arrangement of the capillaries and muscle fibres (to be discussed in the next section). However, the considerable shrinkage incurred during our histological processing made any measurement of SF quite impossible. It should be noted that, except for capillaries on the perimeter of a muscle bundle, the sharing factor can <u>never</u> be less

than two and, in those muscles where the shrinkage was not as great, it was seldom observed to be greater than four. The calculated sharing factors were found to vary approximately between two and three (Table XV), with the mean value being 2.48 ± .07 (SE; n = 19). In the two references in which both N_{VF} and C/F ratio are given, the sharing factors were computed to be 3.79 (Eriksson and Myrhage, 1972) and 2.60 ± .10 (SE; n = 7) (Valdivia, 1958).

Based on these observations, it is very difficult to accept a value of less than 1.0 for the mean C/F ratio in any of the muscles examined in this study. As the sharing factor cannot be less than a value of two, a value as low as 1.0 for the C/F ratio would require that N_{VF} be less than 2.0, a fact not consistent with most observations. It would seem more likely that such low values have resulted from incomplete counting of capillaries, due to technical difficulties in their demonstration. In a similar way, it is difficult to see how the mean value for the C/F ratio could be as high as 3.0, for this implies a mean value of at least six capillaries around <u>each</u> fibre, again contrary to observation. The implications of these restrictions are discussed in the next section on vascular arrangements in skeletal muscle.

III.2.4 Arrangements of capillaries and fibres in transverse sections of muscle

All the models that have been proposed for the arrangement of capillaries and fibres in skeletal muscle require a C/F ratio between 1.0 and 2.0. These models are based on either a square (Hort, 1955, 1968, 1971; Hammersen, 1968) or a hexagonal (Schmidt-Nielsen and Pennycuik, 1961) array of muscle fibres. While Hammersen (1968) indicated that each vessel in the arrangement is located between two adjacent fibres (Figure 16a), Hort (1955) suggested that each is found at the "junction" of four fibres (Figure 16b). Schmidt-Nielsen and Pennycuik (1961) proposed two models, each based on a hexagonal arrangement of capillaries and fibres. In one, that for 'red' muscle, all six junctions are filled by capillaries (Figure 16c), while in the other, for 'white' muscle, they occupy every other corner (Figure 16d). While each of these models represents a regular array of vessels and fibres, many irregular arrangements are possible. One such arrangement which is consistent with the observed data is shown in Figure 16e; it is similar to that proposed for 'white' muscle by Schmidt-Nielsen and Pennycuik, but each vessel is now positioned between two adjacent fibres.

The descriptive parameters for each of the models, and the observed range of values for each parameter, are presented in Table XXI. Since the arrangement shown in Figure 16b requires a sharing factor of four, and that in Figure 16c needs a value of six for the mean number of vessels around each fibre, the observed data rule out completely the possibility of either of these models being applicable to the muscles examined in this study. However, each of the other

Figure 16 - Examples of five possible arrangements of transversely sectioned capillaries and muscle fibres. Models A and B are based on a regular square array of capillaries and muscle fibres. In A, each fibre is placed between two muscle fibres, while in B, each is found at the "vertex" of four neighbouring fibres. Models C and D are based on a regular hexagonal array of capillaries and muscle fibres. In C, each capillary is located at each of the six "junctions", while in D, the capillaries are positioned at every other "vertex". Arrangement E is based on a regular hexagonal array of muscle fibres and an irregular array of capillaries; each fibre has three capillaries around it, and each is "shared" by two adjacent fibres.

A
B
C
D
E

Table XXI

Comparison of limiting values of N_{VF}, SF and C/F ratio for the models of Figure 16 to the observed values

Year	Author(s)	Type of Array	N_{VF}	SF	C/F ratio
1970	Hammersen	square	4	2	2
1955	Hort	square	4	4	1
1961	Schmidt-Nielsen and Pennycuik	hexagonal	6	3	2
1961	Schmidt-Nielsen and Pennycuik	hexagonal	3	3	1
1975	Plyley and Groom	hexagonal	3	2	1.5
1977	Plyley	irregular+	3-4	2-3	1-2

+observed range of values indicates that one particular array does not apply to all muscles

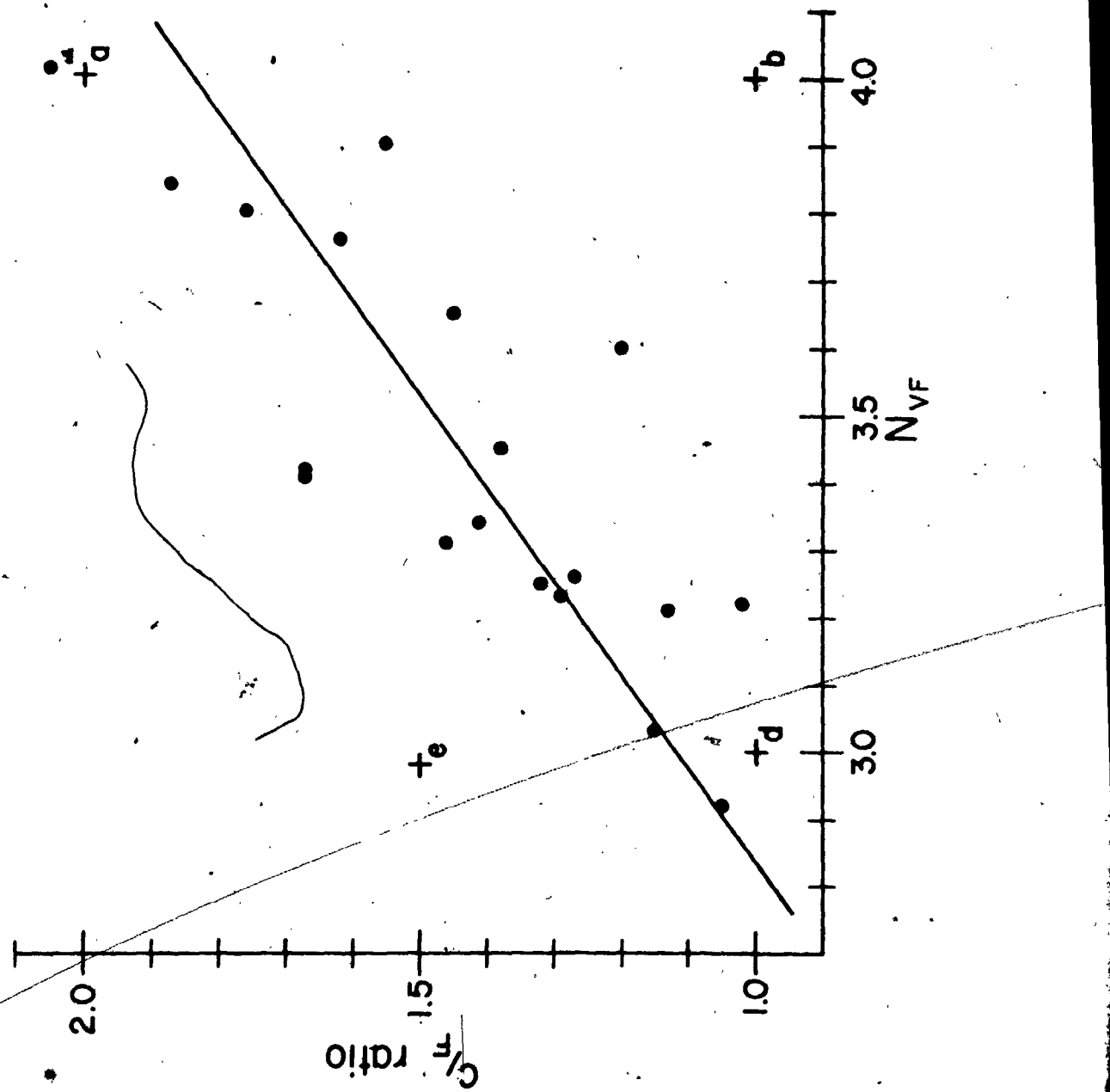
models may well apply on an overall statistical basis.

Further insight into modelling the capillary-muscle fibre arrangement may be gained by considering the relationship between the C/F ratio and N_{VF}. In Figure 17, these variables have been plotted both for the experimental data (n = 19) and for the models a, b, d, and e of Figure 16. It is apparent that the experimental data are scattered roughly about a line joining the points a and d. In fact, the coefficient of correlation for these data was 0.80, and was found to be highly significant ($p < .001$). Moreover, the regression line does not differ significantly from a line joining the points a and d.

It is clear that for rat soleus (pt 4.01, 2.05), the arrangement of capillaries and fibres is best represented by model a, whereas that for frog sartorius (pt 2.95, 1.05) corresponds fairly closely to model d. The arrangements for the rest of the muscles appear to form a series of transitional models between these two extremes. As models b, c and e (Figure 16) are at variance with the experimental observations, they were rejected as possible arrangements describing the capillary-muscle fibre relationship. The specific arrangement describing this relationship appears to differ from one muscle to another in some systematic way. The "average" arrangement for any muscle can be said to be equivalent to (x% model A) + ((100-x)% model D). The factor(s) which influence the specific arrangement in any given muscle have yet to be found, although further analysis is underway in this regard.

Figure 17 - Mean capillary/fibre ratio versus the mean number of capillaries found around each fibre from 19 species of animals, including mammals, amphibians and birds. Points Points a, b, d and e (+) are the corresponding values for the arrangements described in Figure 16. The line represents the least squares regression of the data, and is statistically significant ($r = .80$; $p < .001$). The regression equation is

C/F ratio = $0.74\ N_{VF} - 1.12$.


+a
+b
+c
+d
+e
4.0
3.5
3.0
N_{VF}
2.0
1.5
1.0
C_T ratio

III.2.5 The capillary supply of 'red' and 'white' muscle

Striated muscle is composed, in many cases, of varying proportions of red, white and intermediate fibres. Each type of fibre exhibits distinctive histochemical, biochemical and contractile characteristics (section I.3). Many reports in the literature either suggest, without support of quantitative measurements (Barets, 1952; Henneman and Olson, 1965; Romanul, 1965; Bone, 1966; Reis and Wooten, 1970), or else affirm (Stoel, 1925; Smith and Giovacchini, 1956; Valdivia and co-workers, 1958, 1960; Schmidt-Nielsen and Pennycuik, 1961; Nishiyama, 1965; Carrow et al, 1967; Mai et al, 1970) that the vascularity in red muscle is greater than that in white muscle. This view, however, is not supported by the studies of Duyff and Bouman (1927) or Eriksson and Myrhage (1972), who indicate no clear-cut differences in vascular supply between the two types of muscle.

The present study was not directed toward a comparison of the capillary distribution in red versus white muscle, nor, indeed, was any attempt made to differentiate the composition of the various muscles used in the study. However, recent reports (Ariano et al, 1973; Taylor et al, 1973) have presented the percentage distributions of fibre types in several of the muscles employed in this study (Table XXII).

On the basis of these reports, it is clear that the soleus in these three species represents an almost pure preparation of 'red' muscle (B fibres). The other muscles consist of mainly A and C fibres, (average composition: 61% A, 8% B, 31% C), and thus, represents predominantly 'white' muscle. The mean values of C/F ratio

Table XXII

Reported distributions of the various types of fibres in nine of the muscles used in this study

Animal, muscle	Percentage of each fibre type		
	A	B	C
Cat			
gastroc.	66	18	16
soleus	0	100	0
masseter+	82	10	8
Rat			
gastroc.	58	5	37
soleus	0	84	16
Guinea pig			
gastroc.	56	12	32
soleus	0	100	0
tibial. ant.	50	4	46
gracilis	56	0	44

+ from Taylor et al. (1973)

and N_{VF} found in this study for these two groups of muscles were not significantly different (Table XXIII). This result is somewhat surprising in view of the consensus to the contrary presented by reports in the literature. A careful review of these studies, however, reveals several possible reasons for the discrepancy. The unacceptably low C/F ratios presented by Stoel (1925), Smith and Giovacchini (1956) and Nishiyama (1965) for 'white' muscle are undoubtedly the result of inadequate filling of the vascular bed and/or incomplete counting of capillaries (sections I.6 and I.7). The papers of Valdivia (1958) and Carrow et al (1967) report values of 3.0 and 2.65 for the C/F ratio of 'red' muscle. Even if each capillary is shared by only two fibres (ie. the absolute minimum), these values imply that at least six capillaries surround each muscle fibre, completely contrary to the observed results. Schmidt-Nielsen and Pennycuik (1961) begin their analysis by stating "for this purpose we defined as red fibres those that had approximately two capillaries per fibre, and as white those that had one capillary per fibre". It is no wonder, therefore, that their results indicate a higher capillary supply for 'red' than for 'white' muscle!

It appears that the issue concerning the vascular supply of 'red' versus 'white' muscle is by no means resolved. Although the data from the present study suggest that there is little or no difference between the two, a thorough study of the capillary distribution with respect to the various fibre types (ie. A, B and C fibres) is sorely needed.

Table XXIII

Statistical analysis of the values of N_{VF} and C/F ratio for the nine muscles of Table XXII, grossly divided into predominantly 'red' and predominantly 'white'

Parameter	'Red'	'White'	t-value	df	p-value
N_{VF} $\bar{x}$	3.69	3.49	.967	7	> .3
$\pm$ SE	.22	.10			
C/F ratio $\bar{x}$	1.69	1.48	1.021	7	> .3
$\pm$ SE	.23	.10			

III.2.6 Comment

Although the muscles examined in this study varied in diameter by a ratio of 5:1, the mean number of capillaries found around the perimeter of each fibre was quite consistent (Figure 18). These results suggest that three to four capillaries around a fibre may constitute an optimum number of vessels for supplying oxygen to the fibres of skeletal muscle. Clearly, further experiments, on a wider range of species, must be done to determine if this represents some underlying principle in the design of the vascular architecture in this tissue.

Figure 18 - Mean number of capillaries around the perimeter of muscle fibres from cat and frog. In spite of a 23-fold range in the mean cross-sectional area of the fibres, N_{VF} remains relatively constant over a small range of values (see Table XIV).

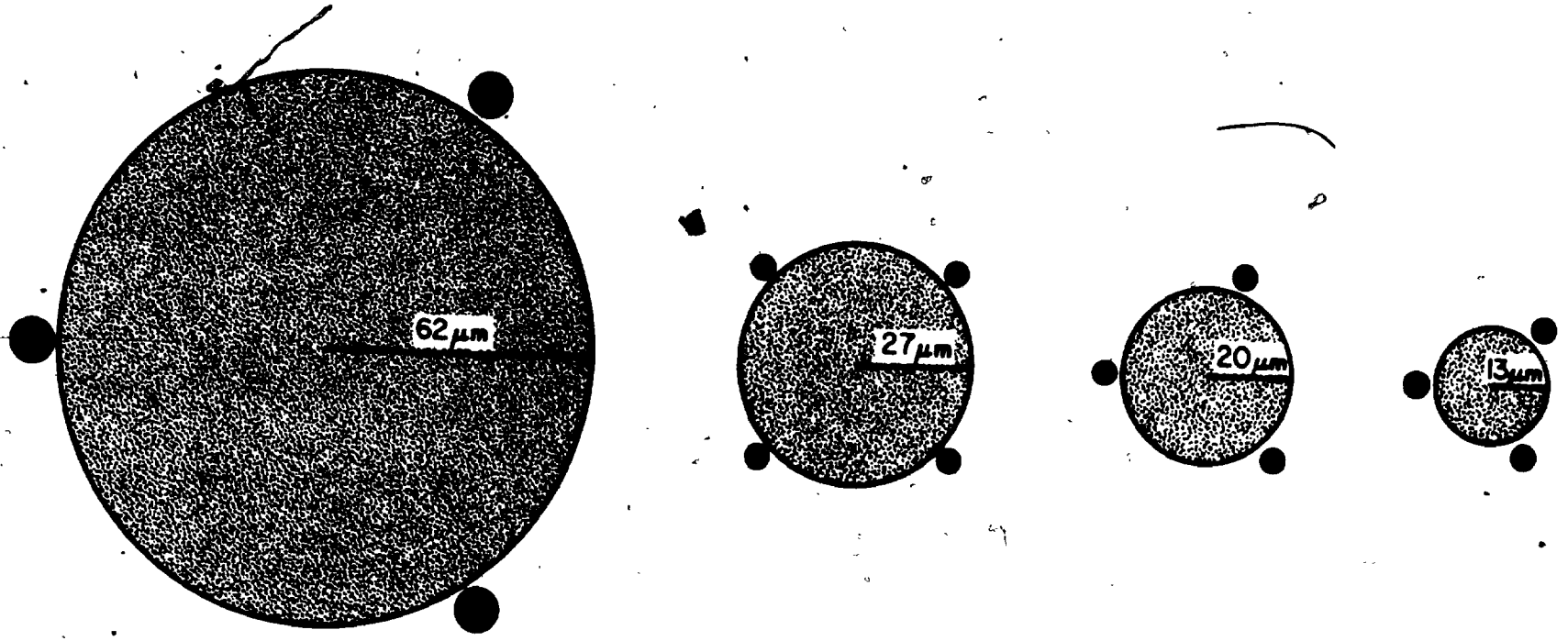
Legend:

F - frog gastrocnemius

CS - cat soleus

CM - cat masseter

CT - cat tongue



$N_{VF.}$	3.03	3.80	3.21	3.23
	F.	C.S.	C.M.	C.T.

The heavens themselves, the planets,
and this centre
Observe degree, priority, and place,
Insisture, course, proportion,
season, form,
Office, and custom, in all line of order.

(William Shakespeare)

IV. ANALYSIS OF THE CAPILLARY BRANCHING PATTERN IN 'LONGITUDINAL SECTIONS' OF STRIATED MUSCLE

IV.1 Results

IV.1.1. Arterial inputs, venous outflows and the direction of flow

The large composite photograph (Figure 10) shows the under-surface of the muscle; X and Y denote the distal and proximal ends respectively. Major vessels enter the muscle at each end and at the midpoint, then run across the muscle obliquely.

As the vascular pattern was very similar from muscle to muscle, comparison of completely filled muscles with others injected only to the level of the capillaries (Figures 10 and 11), enabled a clear distinction to be made between afferent and efferent vessels in the fully injected muscles.

Observations of red cell movements in adjacent capillaries on the surface of the muscle indicated that flow is mostly concurrent, which is consistent with the observations of Bassingthwaighte et al (1974). However, such observations are restricted to surface vessels only and therefore are not necessarily indicative of flow in deeper layers of the muscle.

IV.1.2 Capillary lengths, segment lengths and number of segments per length of capillary

Seven areas from two different muscle preparations were selected for detailed analysis and enlarged composite photomicrographs were prepared for each (Figure 12). In these enlarged areas, it was observed that capillaries from each arteriole (A) run both to the left and to the right, and that at each venule capillaries enter in the same manner. The distance between an arteriole and a venule was spanned by a highly intricate meshwork of capillaries, providing a number of possible routes (paths) for any red cell entering the network from the arteriole. Each of these paths was composed of a number of segments (range 1 to 9)(Figure 19) whose lengths ranged from 0.08 to 3.26 mm (Figure 20). The length of each possible route was determined by summing the lengths of its constituent segments, and was found to vary between 1.08 and 7.14 mm (Figure 21). In all, 620 segments comprising 238 distinct pathways were measured; the average pathway was found to be 3.58 ± 1.64 mm long and composed of 4.44 ± 1.84 segments, each $0.85 \pm .60$ mm long (means $\pm$ SD).

The actual number of segments making up any given path was found to be highly dependent on the length of the path in question (Figure 22).

IV.1.3 Possible pathways available

The total number of capillaries connected together at each point of capillary anastomosis was subdivided into two categories depending upon their history of previous interaction. Any capillary

Figure 19 - Frequency distribution of the number of segments comprising the various routes from arteriole to venule. As each pathway must be composed of at least one segment, the number of segments per path will depend on the number of "interior" branching points along the capillary and, in fact, will be equal to the number of branch points plus one.

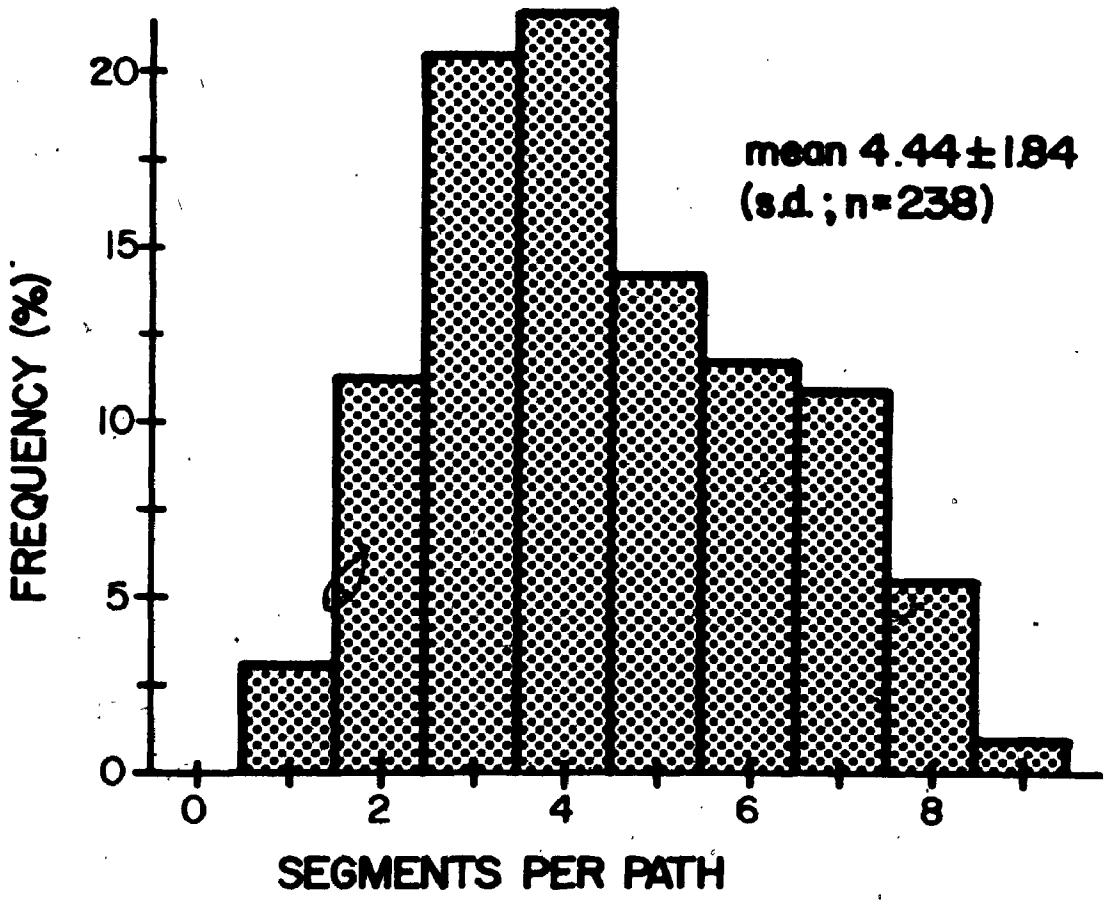

20
15
10
5
0
FREQUENCY (%)
mean 4.44±1.84
(s.d.; n=238)
0
2
4
6
8
SEGMENTS PER PATH

Figure 20 - Frequency distribution of capillary segment lengths, ie. the distribution of distances between branching points along the length of a number of capillaries. The distribution is exponential ($x^2 = 8.24$; degrees of freedom = 5; $p < .20$).

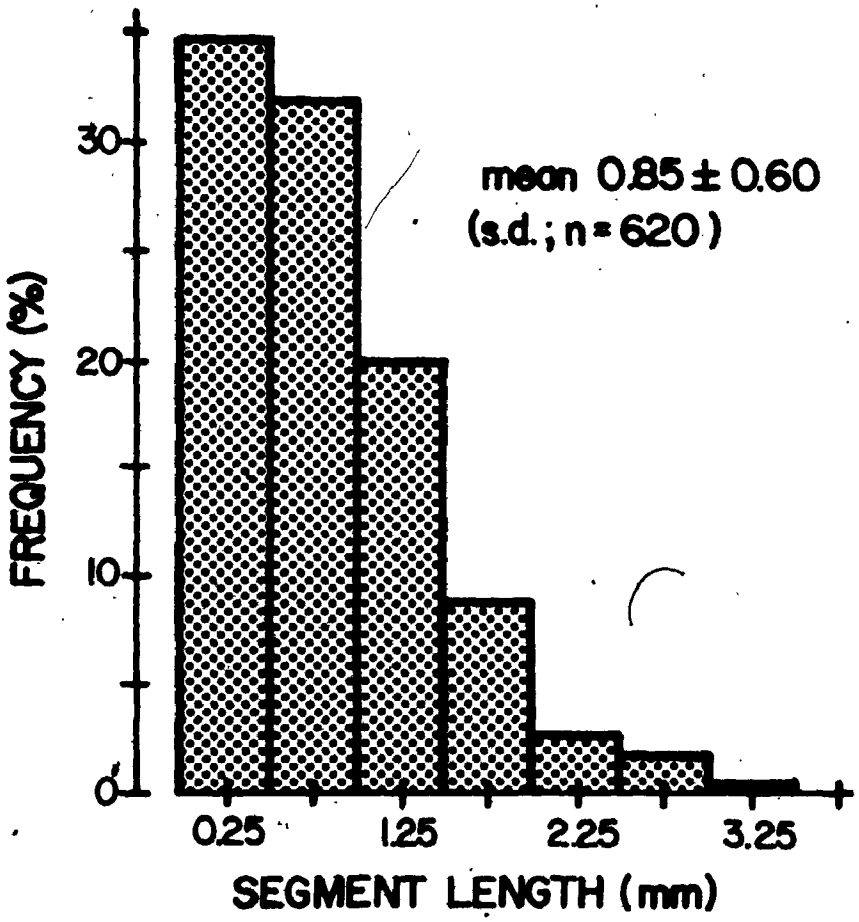

mean 0.85 ± 0.60
(s.d.; n = 620)
FREQUENCY (%)
30
20
10
0
0.25
1.25
2.25
3.25
SEGMENT LENGTH (mm)

Figure 21 - Frequency distribution of capillary path lengths, ie. the arithmetic sum of the lengths of those capillary segments forming any pathway from arteriole to venule. The distribution is Gaussian (x^2 = 4.80; degrees of freedom = 4; p = .30).

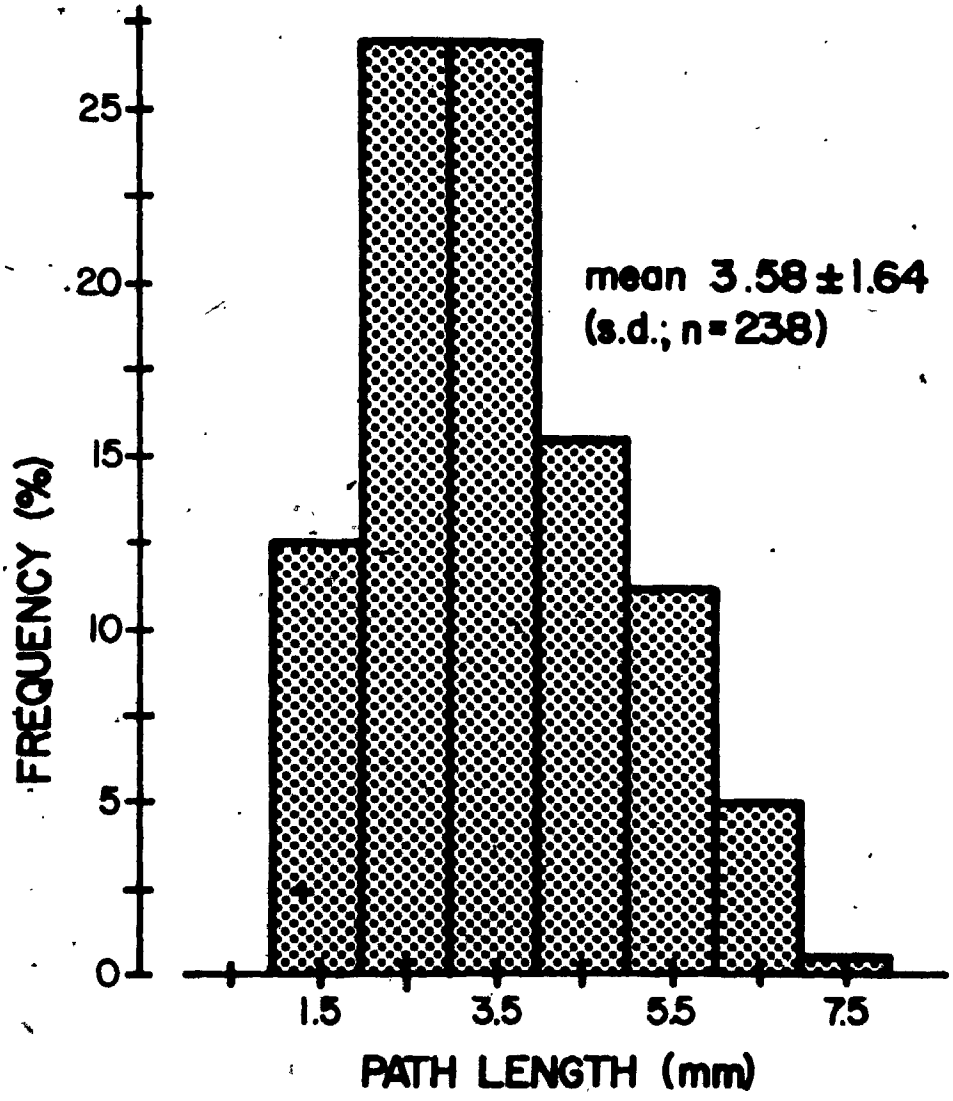

25
20
15
10
5
0
FREQUENCY (%)
mean 3.58 ± 1.64
(s.d.; n = 238)
1.5
3.5
5.5
7.5
PATH LENGTH (mm)

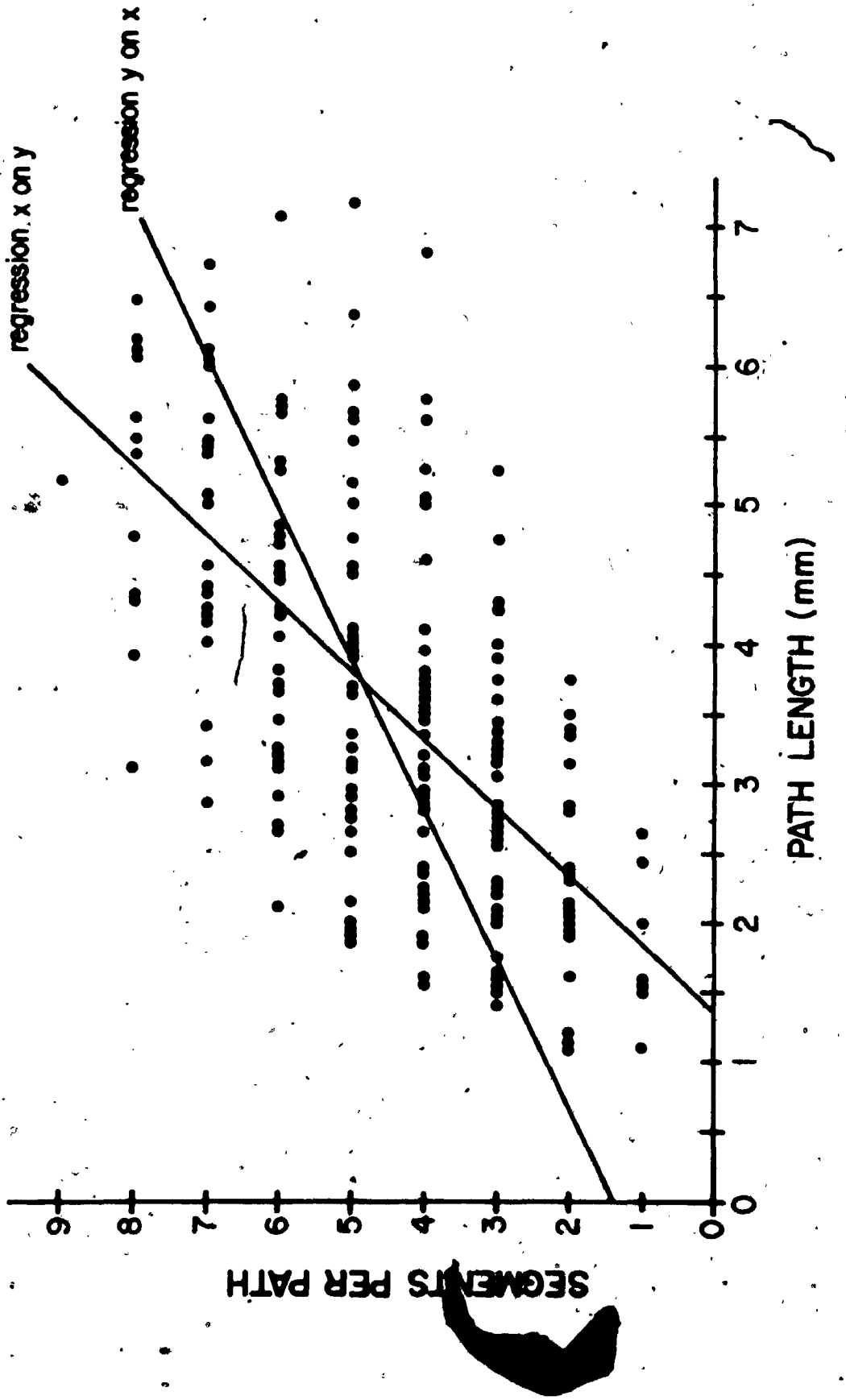
Figure 22 - Scatter graph of the number of segments per capillary versus the length of the capillary path. As the dependence of one variable on the other is unclear, both regression lines are given ($r = 0.64$; $n = 238$; $p < 0.001$). The regression equations are:

y on x: number of segments per capillary =

1.4 + [0.86 · path length (mm)]

x on y: length of capillary path (mm) =

1.4 + [0.48 · number of segments per path].


regression x on y
regression y on x
SEGMENTS PER PATH
PATH LENGTH (mm)
0
1
2
3
4
5
6
7
8
9

which had been connected to the reference vessel at an earlier branch point was classified as "rejoined", while the others, those having no previous interconnections with the reference path, were termed "new".

The numbers of new, rejoined and total capillaries connected varied from branch point to branch point (Figure 23). While the number of "new" capillaries fell from an initial value of 1.7 at b_o to 1.1 at b_1 and levelled off at a value of 0.75 (approx.) at b_3, the number of "rejoined" vessels increased from zero at b_o to a plateau value of 0.25 (approx.) at b_2. Thus the total number of capillaries (= new + rejoined + 1 for the reference vessel) connected at each branch point decreased from an initial value of 2.7 at b_o to 2.0 (approx.) at b_3 and subsequent branch points (Figure 23).

The branching of the network was mainly divergent at the initial branch points, ie. b_o and b_1, but became increasingly more convergent after b_2, and levelled off at a value of 75% convergent branching at b_4 (Figure 24).

While the mean included angle, (Figure 14) between divergent branches, Θ, was found to be 60.1 $\pm$ 28.3° (SD; n = 85), that for convergent branches, ϕ, amounted to 58.3 $\pm$ 24.9° (SD; n = 81).

IV.2 Discussion

IV.2.1 Capillary segment and path length

The various reported values for capillary length are presented in Appendix I. Rous et al (1930) and Smith and Rous (1931) report values of one to two mm for capillary length in frog sartorius, and

Figure 23 - The number of capillary routes joined to the reference pathway, as a function of branch point number down the length of the reference vessel. Each of the 55 distinct routes from arteriole to venule was taken in turn as the reference vessel and the connections to other pathways analyzed as follows:

(i) new capillaries - any pathway being connected to the reference pathway for the first time.

(ii) rejoined capillaries - those pathways which had been connected to the reference pathway at some previous branching point.

The total number of pathways connected at any branch point equals the number of new pathways + the number of rejoined pathways + one (for the continuation of the reference pathway itself). "N" is the number of pathways studied which had the indicated number of branch points, ie. while two of the 55 pathways had only one connection to other routes, there were two that had as many as seven branchings to other pathways. Values are means $\pm$ S.E.

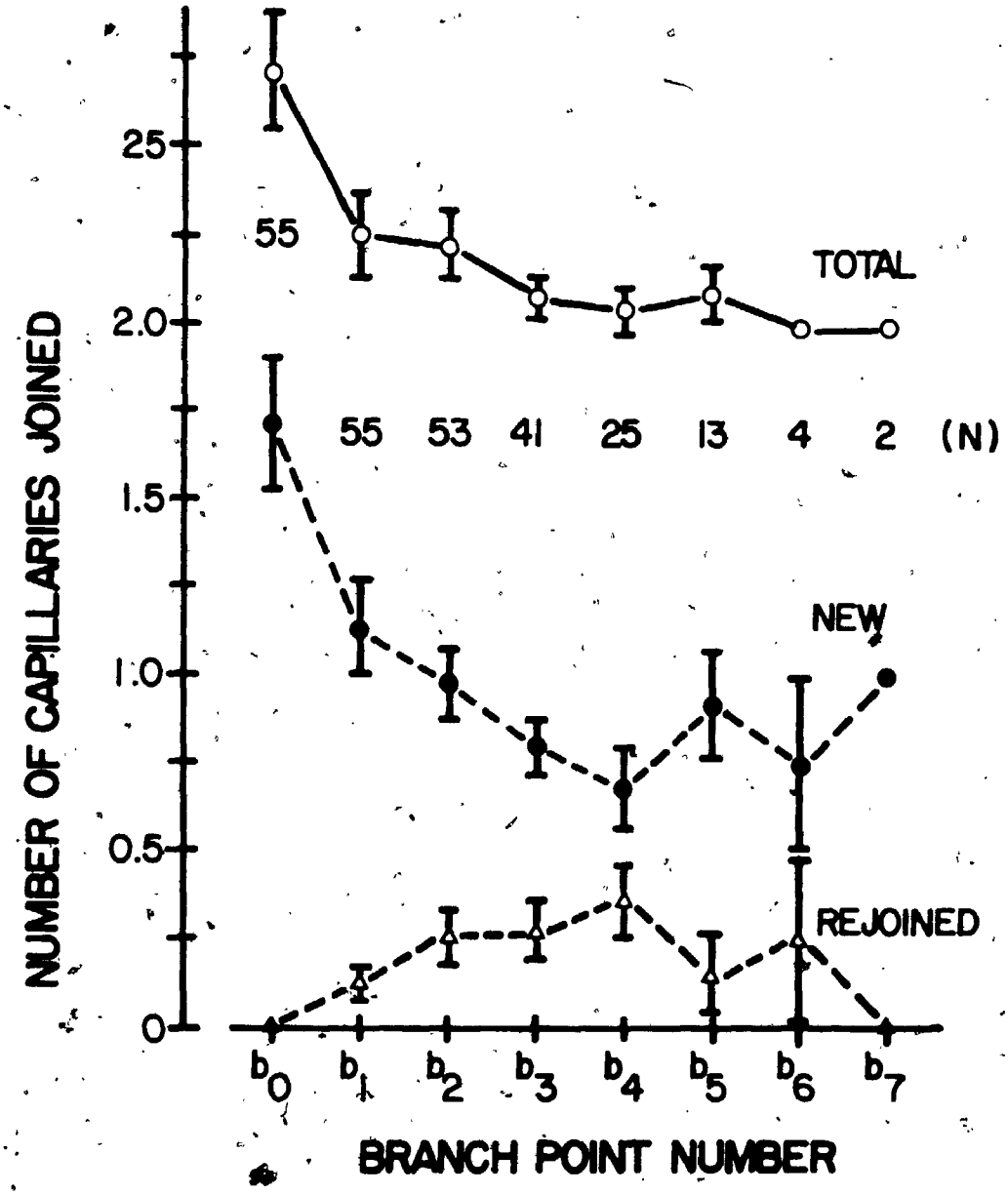
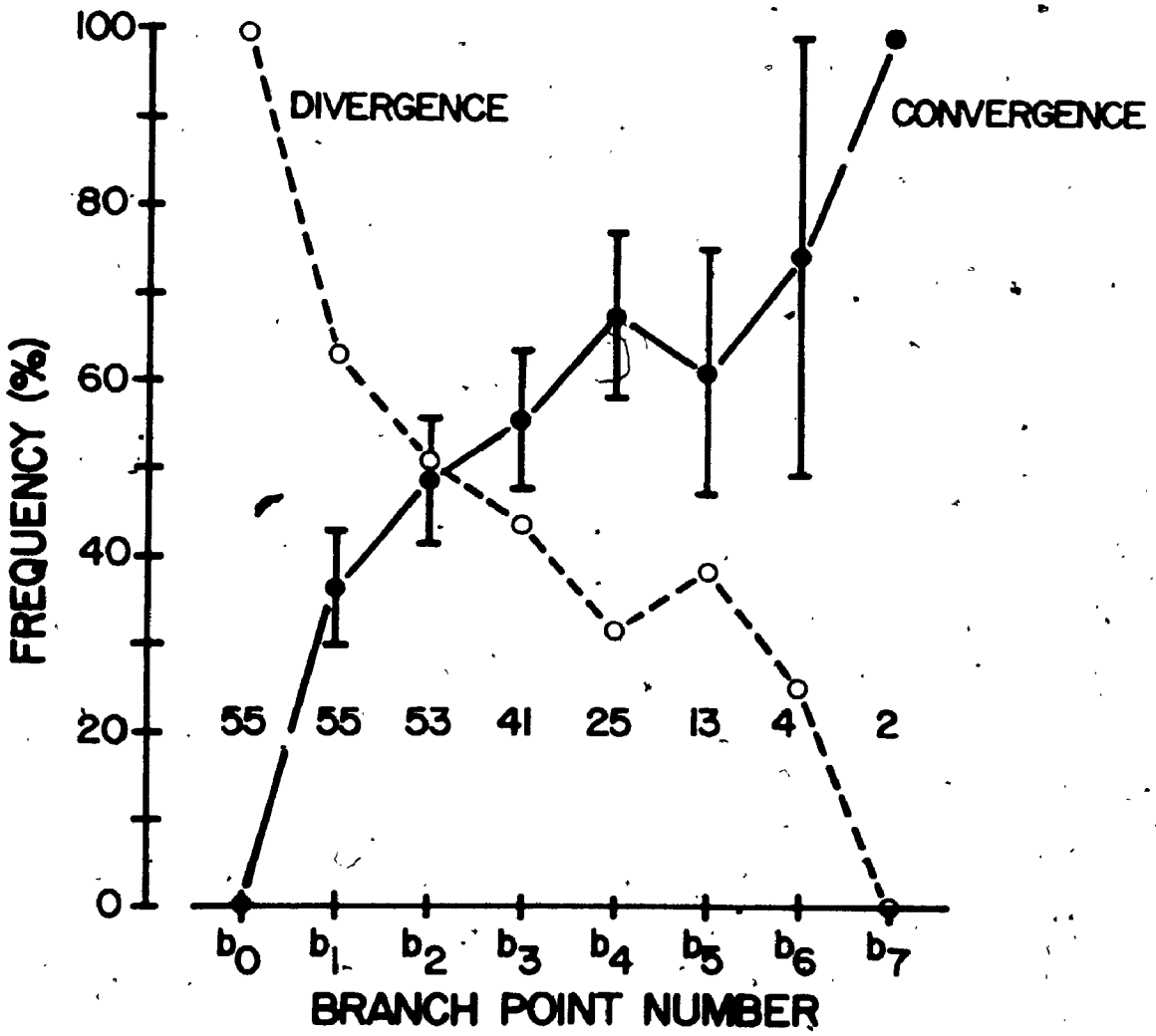

NUMBER OF CAPILLARIES JOINED
25
2.0
1.5
1.0
0.5
0
55
TOTAL
55 53 41 25 13 4 2 (N)
NEW
REJOINED
b_0 b_1 b_2 b_3 b_4 b_5 b_6 b_7
BRANCH POINT NUMBER

Figure 24 - Frequency of divergent and convergent branching as a function of branch point number down the length of the reference pathway. Divergent branches are defined as branching away from the direction of flow, and convergent branches as combining of two concurrent segments (see Figure 14). "N" is the number of pathways studied which had the indicated number of branch points, ie. while two of the 55 pathways had only one connection to other routes (both convergent), there were two that had as many as seven branchings to other pathways.

Note: divergent branching does not necessarily indicate that the anastomosis was to a "new" capillary, nor does convergence imply "rejoining". Both "new" and "rejoined" capillaries can result from either convergent or divergent branching (see Figure 14).


100
80
60
40
20
0
FREQUENCY (%)
DIVERGENCE
CONVERGENCE
55
55
53
41
25
13
4
2
b_0
b_1
b_2
b_3
b_4
b_5
b_6
b_7
BRANCH POINT NUMBER

approximately 0.50 mm in mammalian muscle. Steudel (1938), studying the vascular supply of amphibian muscle, reported mean values ranging from 0.72 to 1.58 mm. Nikiforova and Shoshenko (1963) found capillaries ranging in length from 0.25 to 1.50 mm in the muscles of the common pond frog. In mammalian muscle, the reported values vary from 0.3 to 3.4 mm (Table XXIV), and include values ranging from 0.4 to 3.4 mm for human muscle. In one study (Savaev, 1973), the capillary length was shown to be age-dependent, averaging 0.3 to 0.5 mm in both foetal and newborn muscle, and 0.4 to 3.4 mm in adult muscle.

To my knowledge, the only systematic study of capillary length in skeletal muscle is that of Ivanova (1973). She reports mean values of: 1.237 mm for fish and amphibians, 0.685 mm for reptiles, 0.538 mm for birds and 0.462 mm for mammals (the mean values were calculated by the author).

Some authors, having noted the "segmental" nature of the capillary network, measured the lengths of segments within the network. Steudel (1938) reported values ranging from 0.309 mm in the sartorius to 0.555 mm in the submaxillaris. The quotient of (mean capillary length)/(mean segment length) yields the number of segments making up the path, and for these two muscles, the values would be 2.33 and 2.09 respectively. Nikiforova and Shoshenko (1963) state that "a few intercapillary bridges, not more than 200 μ in length, are present between the capillaries of the same arteriole and also between the capillaries of different arterioles" but no indication of segment length is given.

Reported values of segment length in mammalian muscle range from 0.01 to 0.21 mm (Honig et al 1970; Smaje et al, 1970; Eriksson and Myrhage, 1972; Nikolov and Schumacher, 1973). The corresponding number of segments per path length (equal to the quotient of path length/segment length) varies from 2.3 to 8.0 (Appendix I).

Part of the discrepancy between the results of the present study and those of Rous et al (1930), Smith and Rous (1931), and Steudel (1938), is explained on the basis of a different definition of capillary length. In agreement with Eriksson and Myrhage (1972), the beginning of a capillary was taken to be the point of last division of the arteriole (Figure 12), and the end of the capillary to be the point where it joins another capillary to form a venule or empties directly into a significantly larger venule at right angles. In terms of Figure 14, Rous et al (1930), Smith and Rous (1931) and Steudel (1938) have measured the distance from branch point b_1 to the last branch point before the junction with the venule. Steudel's paper indicates that inclusion of both end-segments would increase his measurements of capillary length by 1.094 mm. However, the resulting lengths (2.493 mm for R. esculenta and 2.237 mm for R. temporia) are still much lower than my own values. However, the measurements of path length here reported include all combinations of longitudinal and transverse segments along which a red cell might flow in passing from arteriole to venule; the path lengths will thus be greater than the straight-line distance between arteriole and venule by a distance dependent on the number of anastomoses between

Table XXIV

Reported values of capillary length
in mammalian skeletal muscle

Year	Author(s)	Animal, muscle	Capillary length (mm)
1968	Markizow	human	0.60-0.70
1970	Smaje et al	rat cremaster	0.615
1970	Hammersen	rabbit rectus abdominus	0.75-1.30
		rabbit adductor magnus	0.30-0.75
1970	Honig	rat gracilis	0.40-0.50
1972	Eriksson and Myrhage	cat tenuissimus	1.015
1973	Mankovska	dolphin spinalis	0.42
1973	Sabaev	human (adult)	0.40-3.4
		human (newborn)	0.30-0.50
1973	Nikolov and Schumacher	dog tongue	0.12-0.15
1976	Hudlicka and Myrhage	rat extensor hallucis proprius	0.55
		rabbit tenuissimus	1.12

longitudinal segments. It appears that the aforementioned authors measured only straight line distances. The remaining difference is most likely the result of considerable shrinkage of the tissue during the dehydration and embedding procedures employed by Steudel.

Measurement of 20 capillary paths in tenuissimus of cat yielded a mean path length of 1.06 ± 0.044 mm (SE; n = 20), which compares quite closely to the value 1.015 ± 0.016 mm (SE; n = 1275) reported by Eriksson and Myrhage (1972) and Eriksson et al (1973). Now even a cursory comparison under the dissecting microscope shows that the distance from arteriole to venule in sartorius of frog is roughly three times as long as that in tenuissimus of cat. Thus the value of the mean path length, 3.58 ± 1.64 mm (SD; n = 238), for sartorius of frog appears perfectly reasonable. It is therefore very likely that the discrepancy between my own results and those of Rous et al (1930), Smith and Rous (1931) and Steudel (1938) is the result of differences in defining and measuring the capillary length. Additional evidence for this conclusion can be drawn from a direct comparison of my results with those of Steudel. The mean values for segment length and path length are 1.52 and 1.51 times greater than the respective mean values reported by Steudel (note: for comparative purposes, the lengths of the end segments were added to Steudel's reported values of capillary length). This indicates that the use of a similar definition of capillary length would result in mean values differing only by a factor equal to the shrinkage incurred during the histological processing.

This same problem, that of defining what to include in measuring capillary length, also affects the number of reported

segments making up the capillary length, ie. a value of two must be added to each of values calculated from Steudel's measurements. The addition of these two segments per path show that in frog muscle, the average number of segments per path is 4.21 as compared to the value 4.44 ± 1.84 (SD; n = 238) found in the present study.

IV.2.2 Distributions of capillary length parameters

The frequency distribution of capillary segment lengths (Figure 20) was definitely exponential in character (χ^2 = 8.24; degrees of freedom = 5; p = 0.15). The data clearly shows the enormous range of segment lengths encountered, from 3.26 mm down to 0.08 mm, ie. 40:1. One-third of these segments were less than 0.5 mm long and two-thirds less than 1.0 mm. In contrast to this, the frequency distribution of the total path lengths (Figure 21) was bell-shaped and near normal with slight skewness in the direction of longer paths (χ^2 = 4.80; degrees of freedom = 4; p = 0.30). While the range of segment lengths was great, the total path lengths varied only by a factor of seven. These path lengths represent the length distribution of all the transit paths for blood through the capillary network.

To put these measurements into perspective, and to facilitate comparison of muscles from different species, the segment and path lengths should be expressed in terms of a common denominator, namely the fibre diameter. Since the mean fibre diameter was found to be 0.067 ± 0.019 mm (SD; n = 83), the mean segment length would be 12.6 ± 9 fibre diameters (range 1.2 to 48.5) and the mean path length 53.5 ± 24.5, ranging from 16 to 107 fibre diameters.

In spite of the very large variation in segment length, the number of segments making up any given path was well correlated with the length of that particular path (Figure 22). As the interdependence of the two variables is uncertain, both regression equations have been given ($r = 0.64$; $n = 238$; $p < 0.001$). Regardless of the dependency, the correlation suggests that, on the whole, longer paths are achieved by adding further segments to the path, rather than by simply increasing the length of the segments within the path. Such a finding raises the question: is there some fundamental reason why mean segment length should not exceed a certain limiting value?

The frequency distribution of the number of segments per path (Figure 19) shows a very large variance (equal to 79% of the mean value), as would be expected for a Poisson distribution. However, the latter refers only to events, in space or time, and requires that there be a finite chance of no event occurring. A segment can hardly be regarded as an event and, by definition, each path must consist of at least one segment. However, the number of branch points found between the two ends of the capillary will always be one less than the number of segments. Thus, subtracting a value of one from the number of segments per path, yields the number of branch points per path. Now a branch point may indeed be considered as a spatial event, and if the probability of its occurring at any given point along the path be small, then the frequency distribution of the number of branch points per path should be Poisson in nature. A reconsideration of the data of Figure 19 in this way shows that the numbers of branch points per path range from zero to eight with a mean value of

3.44 ± 1.84 (SD; n = 238). The ratio of the variance to the mean is 0.98, and the distribution corresponds very closely to a Poisson distribution having a mean of 3.44 ($\chi^2 = 3.00$; degrees of freedon = 5; $p = 0.70$). The only question regarding this treatment of the data is that the total paths should be of constant length. However, as the path lengths were found to have a Gaussian distribution about a mean value, and the number of paths measured was very large (n = 238), the distribution of branch points per path should correspond rather closely to that obtained for vessels having a path length equal to the mean path length (3.58 mm).

Armed with this knowledge, it is now possible to reconsider the character of the distribution of segment lengths (Figure 20). If the frequency of events in space or time follows a Poisson distribution, the intervals between events are expected to follow an exponential distribution (Colquhoun, 1971). The frequency of intervals lying between x_1 and x_2 will be given by $(e^{-\lambda x_1} - e^{-\lambda x_2})$, where λ is the reciprocal of the average interval. Since the distribution of the number of branch points per path is indeed Poisson, the distribution of segment lengths should therefore be exponential. This was found to be the case, the rate constant being 1.18 mm^{-1} (1/0.85 mm). A comparison of the frequencies of segment length determined experimentally with those calculated using this rate constant, shows that the expected frequencies correspond fairly closely to the observed frequencies ($\chi^2 = 8.24$; degrees of freedom = 5; $p = 0.15$). This confirms, indirectly, that the underlying distribution of branch points per path is indeed Poisson.

The data on the frequency of branching and the distribution of segment length thus support the idea that capillary branch points (ie. anastomoses) are randomly distributed along the length of the vessels, there being a small but constant probability of branching at every point along the vessel. It follows then that the number of branch points should be proportional to the path length (as was found: Figure 22). Thus, when taken together, Figures 19-22, reinforce the same idea, that being, that in skeletal muscle the points of anastomoses are randomly distributed along the length of the capillary. This hypothesis carries the implication that the capillary anastomoses must have developed, during growth, on a purely random basis. While the lengths of the segments comprising any path were found to vary considerably (0.08 to 3.26 mm), the range of path length was quite small (1.08 to 7.14 mm). This is to be expected, for path length is governed by the distance between arterioles and venules, and thus will be constrained by the organization of the major vessels within the muscle (Figure 10).

IV.2.3 Analysis of the observed pattern of anastomoses

To appreciate the distinguishing features of the capillary network, it is perhaps useful to contrast the branching pattern of its numerous anastomoses with that of the simplest branching system, one in which each successive segment gives rise to two branches, ie. a constant value of 2.0 in Figure 23. It is evident that in the capillary network, the total number of capillaries joined at successive branch points approaches this value of 2.0 from branch point b_3 onwards; however, at b_1 and b_2, and particularly at b_0, the

mean value is significantly greater than 2.0. Moreover, since at each branch point one of the connected vessels is, by definition, the reference vessel itself (see Methods and Analysis), the number of new capillaries joined at each branch point in the simple bifurcating system will be one, ie. 1.0 on Figure 23. The pattern seen in the capillary network differs quite significantly from this; the number of new capillaries joined at b_o is 1.7, decreases rapidly through b_1, b_2, to a roughly constant value of 0.75 at b_3 and later branch points. The difference between this plateau value of 0.75 and the value 1.0 is due to the presence of rejoined capillaries at each branch point, which rises from a value of zero at b_o (by definition) to 0.25 (approx.) at b_2 and later branch points.

The simple bifurcating system is used by the body in the conducting blood vessels and airways where no exchange of diffusible substances is required, but considerable changes of both diameter and length must occur between successive orders to ensure that the hydrostatic pressure gradient is as uniform as possible. The functions performed by such a system require that it "fan out" repeatedly. However, in the capillary network, where the main function is exchange of solutes, a large surface area to volume ratio and a constant mean flow velocity are required at all levels of branching. This dictates that the system should not "spread out" but remain confined to a rather limited area and further, that the mean diameter and length in successive segments should remain constant (mean capillary diameter was found to be 12.5 ± 2.8 μm (SD; n = 38)).

The observed branching pattern has the following characteristics: (1) the greatest divergence occurs at b_o, (2) interconnections exist between capillaries deriving from the same arteriole, and (3) following the initial branching (b_o), convergences (of which all are not necessarily rejoined vessels) occur with increasing frequency. The effect of such a branching pattern is that the system diverges immediately at b_o and then, through interconnecting paths, passes to the venule without further divergence. This leads to a more compact network without areas of insufficient supply.

IV.2.4 Modelling the capillary network

Any study of the geometry of the capillary network in the longitudinal direction would have as an objective, the formulation of a proper three-dimensional model. In principle this could be achieved using the present data since, by virtue of the axial symmetry which exists in skeletal muscle, the capillary geometry in two longitudinal planes, one parallel and the other normal to the muscle surface, should be the same. Although this task appears formidable, it is fair to say that recent advances in the understanding of O_2 transport to tissue have been limited chiefly by a lack of the basic experimental input data, rather than by any lack of sophistication in the mathematical methods available for precise modelling.

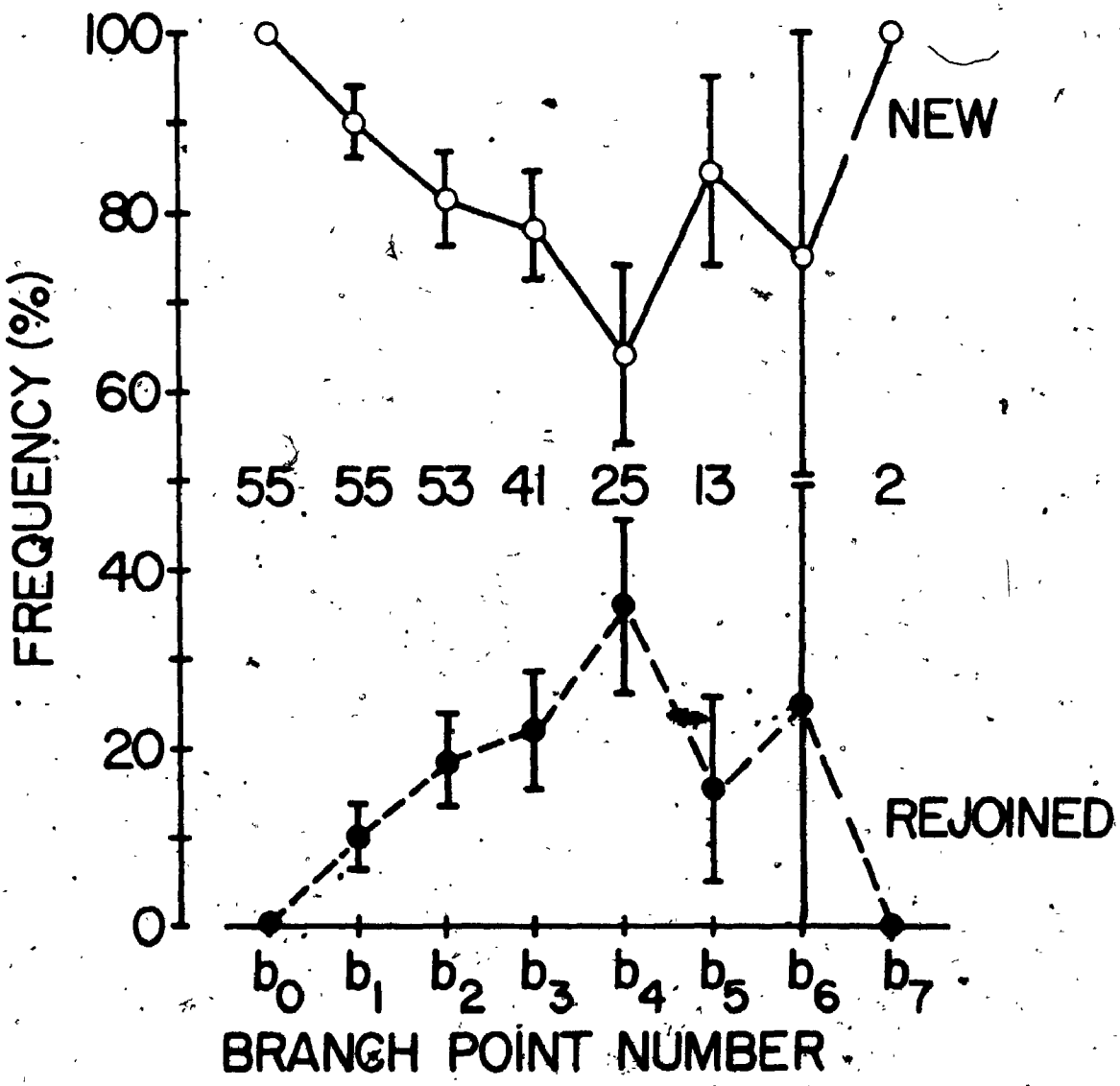
The first model proposed as a basis for O_2 transport to tissue from blood in the capillaries of skeletal muscle was that of Krogh (1919). Here a single, long straight capillary was assumed to be representative of the whole, all interconnections between capillaries

and any inhomogeneity of perfusion being disregarded. For almost 50 years the 'Krogh cylinder' remained the only model available, because solution of the diffusion equation in the analysis of O_2 supply could not be achieved explicitly for more complicated cases. With the advent of high speed digital computers, however, it has become possible to obtain numerical solutions for the distribution of tissue P_{O_2} using more realistic (and thus more complicated) models. That proposed by Grunewald (1968, 1973) assumes four parallel capillary sections with various arrangements of arterial and venous connections; however, it neglects all interconnections between capillaries. The model proposed by Metzger (1973) is based on a regular cubic array of interconnecting capillaries having flow inhomogeneities of 27:1. It would appear to be a very useful model which could be modified fairly readily, on the basis of the quantitative data here presented, to give a more realistic picture of the variation of tissue P_{O_2} in skeletal muscle.

Such modelling of the capillary network would require, however, a knowledge of the <u>percentage</u> of rejoined vessels at each branch point. This is given by the quotient (rejoined capillaries x 100%)/(total capillaries -1), the value unity in the denominator representing the reference vessel. These percentages (Figure 25) rise from a value of zero at b_0 to a plateau value of 25% (approx.) at b_3 and later branch points. The corresponding frequency of new capillaries joined at each branch point of course equals (100 - % rejoins).

Using the above data, it is possible to construct a two-dimensional simulation of a network which has all the branching characteristics of the actual capillary network. The essential

Figure 25 - Frequency of "new" and "rejoined" capillaries as a function of branch point number down the length of the reference pathway. "New" capillaries are pathways being joined to the reference pathway for the first time, while "rejoined" capillaries are those which have been connected to the reference pathway at some earlier branch point. "N" refers to the number of branch points, ie. two of the 55 pathways have only one connection to other pathways, while two others have as many as seven such branching points. Values are means $\pm$ S.E.


100
80
60
40
20
0
FREQUENCY (%)
NEW
REJOINED
55 55 53 41 25 13 2
b_0 b_1 b_2 b_3 b_4 b_5 b_6 b_7
BRANCH POINT NUMBER

information required to construct such a simulation is as follows:

1. the mean number of capillaries arising at each branch point (Figure 23);
2. the numbers of segments per path (Figure 19);
3. the percentage of rejoined capillaries at each branch point (Figure 25);
4. the proportions of convergences and divergences at each branch point (Figure 24);
5. the scaling factors: total path lengths (Figure 21)
   lateral spacing (to be discussed)
   branching angles (see text).

An example of one such simulation, drawn by hand, and constructed according to the above information, is presented in Figure 26, and for comparison a line drawing of an actual network which has been traced from Figure 12. The similarities between the two are obvious.

As mentioned earlier, the depth of focus of the field of view was such that only the capillaries associated with the uppermost layer of muscle fibres could be photographed and measured. In these frogs, the mean fibre diameter was 0.067 ±.019 mm (SD; n = 83), so that the vessels shown in the simulation (Figure 26) are those contained in a layer of this thickness. When viewed in transverse section, three capillaries are found around each fibre, and each vessel is shared by three fibres (section III.1.3). As a consequence of analyzing the three-dimensional structure using two-dimensional photographs, the mean lateral spacing between the capillaries will correspond to

Figure 26 - Two line drawings of the capillary network; above, a network traced from an actual network using microslide projection, and below, a network simulated from the observed data and the rules presented on the preceding page.

Scale: 2.5 cm ≡ 1 mm


A
V


A
V
V
A
A
V

one-half the mean fibre diameter, or more precisely, to one-half the radius of the Krogh cylinder (0.074 mm). Using this information, the simulated network has been drawn to the same scale in the transverse dimension as the actual network.

IV.2.5 Comment

The role or functional significance of the capillary anastomoses is a question of fundamental importance in understanding the microcirculation in skeletal muscle. It is easy to see that they safeguard tissue perfusion by providing alternate pathways through the tissue when capillary segments become blocked, as in the case of blocking by white cells (Eriksson and Lisander, 1972a, b; Olofsson et al, 1973). Such an occurrence would lead to non-uniform perfusion of blood (Johnson, 1970) and clearly, any tissue with non-anastomosing capillaries would be vulnerable. In this way, the anastomoses represent a significant "safety factor". This view has been elaborated with regard to microemboli in myocardial capillaries by Brown (1965).

It might, however, be unwise to assume that this represents the complete answer to the question, for the capillary network appears to be so highly developed as to represent an "over-response" to this particular problem. It is interesting to speculate as to whether these anastomoses may not in some way lead to an improved distribution of blood flow and tissue oxygenation, particularly in working muscle.

"Note that as a rule, simple questions do not have simple answers. In fact, the relation is inverse; the simpler the question, the more complicated the answer is likely to be. To have a simple answer, the question must be complicated, specialized and circumscribed."

(A. C. Burton)

V. GENERAL DISCUSSION AND CONCLUSIONS

The maintenance of an adequate "milieu interieur" for the cells of the body is the result of many physiological systems working in harmony. In the higher life forms, transport of cellular metabolic requirements is achieved by a specialized transport system.

Transport of oxygen to the cells by the cardiovascular system may be discussed under two headings - the determinants of arterial oxygen tension and factors operating at the level of the tissues.

Krogh (1919a, b, 1922) developed the first model describing oxygen diffusion from a capillary to a cell. He reasoned that the oxygen tension, $P_{O_2}(x)$, at a given point, x, within a tissue would be dependent upon: (i) the difference in the partial pressure of oxygen between the capillary and that at point x ($P_{O_2}(c)-P_{O_2}(x)$), (ii) the distance between the capillary and point x, (iii) the radius of the capillary (r_c), (iv) the rate of oxygen consumption by the tissue (q'), and (v) the permeability constant for oxygen in the tissue ($D\cdot\alpha$). The difference in partial pressure will result in a radial gradient out from the capillary. This gradient will be dependent on the rate of tissue metabolism (q'), and the distance (2R) between capillaries being actively perfused at any one time. This corresponds to the case of diffusion from a cylinder, radially outward into a metabolizing tissue; the metabolism is assumed to be constant throughout the tissue, and independent of the

partial pressure of oxygen. This was presented mathematically in equation (2) (Introduction). In equation (2), when $x = R$, $P_{O_2}(x)$ will be a minimum value and the equation becomes:

$$P_{O_2}(c) - P_{O_2}(x) = \frac{q'}{D\alpha}\left[\frac{R^2}{2} \ln \frac{R}{r_c} - \frac{R^2 - r_c^2}{4}\right] \qquad (3)$$

This equation, developed by Erlang for August Krogh in 1919, has been the basis for most computer simulations of oxygen transport dynamics for the last half-century.

Many "refinements" and modifications have been made to the model since its development (see Bassingthwaighte, 1974). Since the advent of high speed digital computers, the effect of "relaxing" certain constraints has been tested quantitatively (Hudson and Cater, 1964; Middleman, 1972). However, each "new" model appears to suffer from over-simplification. Honig and Bourdeau-Martini (1974) state that for the heart, the only two assumptions of importance are: (i) diffusion fields around individual capillaries do not interact and (ii) capillary spacing is uniform throughout the tissue. The first assumption, all-important to simplifying the mathematics of the problem (Honig and Bourdeau-Martini, 1974), is valid only for concurrent flow. While Honig et al (1970) found frequent cases of counter-current flow in rat gracilis, personal observations of the vessels of frog sartorius have yielded fewer instances of such flow. In either case, the frequent inter-capillary anastomoses, as yet not included in any model, may have a considerable effect on this assumption. Although Honig and Bourdeau-Martini (1974) state that

"less than 15% of (the intercapillary) spacings (in rat heart) are greater than 1.2 x the mean" at normal arterial pO_2, such is not the case in skeletal muscle, where distances ranging from "0.1-3 times the 36 μ mean value were found" (Honig et al. 1970, 1971). These findings relegate most commonly used models, based on a Krogh cylinder, or a regular array of Krogh cylinders, to trivial tasks of computer programming.

Only in recent years, have experimentalists, realizing the need for precise anatomical information, begun to analyze quantitatively the organization of the microvascular beds of various tissues. As stated by Wiedeman, who first measured the lengths and diameters of the vessels in the living bat wing in 1962, such measurements are necessary "in defining the proper relationships between the various portions of the vascular bed" (1963).

A knowledge of microvascular topography, with accurate measurements of the components, is fundamental to understanding microcirculatory dynamics. It has been suggested by both Eriksson and Myrhage (1972) and Branemark (1973) that each tissue in the body must be carefully studied "before we can make any attempts at finding some kind of microvascular common denominator". In this regard, several recent studies on quite diverse tissues are very encouraging.

Intaglietta and Zweifach (1971) proposed a model for the vascular bed of the rabbit omentum based on average values from their in vivo measurements. Frasher and Wayland (1972) reported a "repeating modular organization" in the microvasculature of the mesentery of cat. The vessels and vascular connections of the rat

cremaster muscle have been described by Smaje et al. (1970). The microvascular organization of the vessels in the cat tenuissimus was reported by Eriksson and Myrhage (1972) and Eriksson et al (1973). Both Grayson et al (1974) and Bassingthwaighte et al (1974) have analyzed in some detail the composition of the coronary vascular bed. Although Bassingthwaighte et al described two types of capillary anastomoses (Y-junctions and H-connections), little work has been done to describe quantitatively the interconnections of the capillary network in any tissue.

Both theoreticians and experimentalists agree that "the principal obstacle to quantitative analysis of tissue-capillary exchange remains the lack of what Krogh termed 'quantitative anatomy'..." (Honig et al, 1971). Bruley (1973) states that "the greatest problem facing the theoretician and the experimentalist is the establishment of a meaningful geometry and flow pattern....A great amount of work will be necessary to provide the necessary geometric relationships, capillary lengths, diameters and other anatomical parameters needed for precise modelling". Honig and Bourdeau-Martini (1973) agree: "We conclude that progress in O_2 transport is chiefly limited by lack of knowledge of parameters..., it seems likely that exciting new insights can be expected from theoreticians if experimentalists can provide the anatomical and physiological facts." With regard to the coronary circulation in particular, they state, "the principle impediment to quantitative analysis of tissue-capillary exchange is uncertainty about capillary geometry, and the numerical values of physiologic parameters. We still know nothing

of the arrangement of capillaries in three dimensions, except that the distribution is not completely random. Detailed information about capillary arrangement would not have been useful in Krogh's time, because mathematical techniques for modelling the data were unavailable. Today O_2 profiles for any capillary arrangement could be computed were the arrangement known. Other important anatomical unknowns include the degree of alignment of arterial and venous ends of capillaries, the extent of capillary branching, and the frequency distribution of capillary lengths. Physiological unknowns include the extent of interaction of diffusion fields around capillaries, the extent of countercurrent flow, the nature of longitudinal gradients in capillaries and tissue, and the effect of diffusion from pre- and post-capillary vessels on these gradients. Undoubtedly, many of the above factors change under different physiologic conditions..." (Honig and Bourdeau-Martini, 1974).

The present study was undertaken because of the need for a quantitative description of the arrangement of capillaries in skeletal muscle, in hope that such measurements of capillary geometry would lead to a better understanding of oxygen transport and microcirculatory dynamics in this tissue. To obtain the necessary information, the capillary network was analyzed in both transverse and longitudinal section.

It was noted that the classical descriptions of the relation of capillaries to muscle fibres in transverse section, ie. capillary density and capillary:fibre ratio, are subject to many errors, some of which are inherent in the tissue itself. A better

method of describing the relationship is to determine the mean number of capillaries located around the periphery of transversely sectioned muscle fibres. Analysis of seven species (five mammalian, one avian, one amphibian) has shown that the mean number of capillaries found around each fibre lies between three and four; the overall mean value was 3.45 ± .07 (SE; n = 19). However, to describe completely the relationship of the capillaries and fibres, two other parameters, the sharing factor and C/F ratio, should be measured independently. The mean values for these parameters were found to be 2.48 ± .07 (SE) and 1.44 ± .06 (SE) respectively. A knowledge of all three parameters is essential to modelling capillary-muscle fibre arrangement in transverse section. It was found that the muscles here analyzed could not be described solely by either of the "square array" or "hexagonal array" models. In fact, the "average" model would appear to be approximately 50% hexagonal and 50% square array and, the model describing the arrangement for any individual muscle, is composed of varying amounts of each of the two arrays. As yet, the factors dictating which array predominates in any given muscle have yet to be determined; however, one strong possibility appears to be the distribution of fibre sizes found within a muscle.

Analysis of the capillary network in the longitudinal plane has shown the branching pattern to be a complex maze of routes from arteriole to venule. Distinguishing <u>each</u> segment in the network allowed all possible pathways from arteriole to venule to be mapped out. The length of each of these transit paths was determined by summing its component segments. The distributions of the number of branching points in each path and the segment lengths are consistent

3 3

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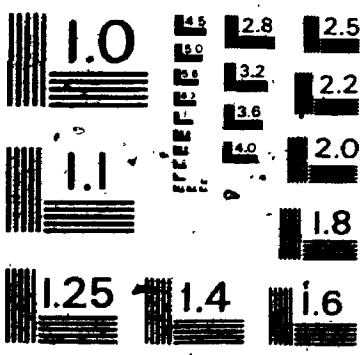


with the hypothesis that the branching of capillaries is random. The mean path length, mean segment length and mean number of branchings per path were 3.58 ± .11 mm (SE; n = 238), 0.85 ± 04 mm (SE; n = 620) and 3.44 ± .12 (SE; n = 238) respectively. The dependency of path length on the number of segments composing the path indicates that to increase a path's length, additional segments are added as opposed to increasing the length of segments within the path.

The interconnections between capillaries were described at each branching according to whether the anastomosis joined the reference pathway to a path which had been connected to the system previously, ie. a "rejoined" pathway or one which was completely separate, ie. a "new" pathway. In addition, a knowledge of the general direction of flow made it possible to distinguish whether connections were convergent or divergent. Analyzing the network in this way, has shown that the capillary branching pattern is one which diverges immediately at the terminal arteriole (branch point b_o) and then, through interconnecting paths, passes to the venule without further divergence (in fact convergence increases). Initially the interconnections must be to new paths; however, at the point where the system becomes equally divergent and convergent (approx. b_2), the number of "new" pathways joined at each branching is approximately three times the number of "rejoined" routes (Figure 25). Yet to be determined is the significance and fundamental importance of the numerous anastomoses observed within the system.

These results provide a frame of reference, ie. what capillaries and connections are present morphologically, for modelling oxygen

transport to muscle, and studying flow patterns in this tissue under various physiological conditions. Furthermore, using this approach, it will now be possible to compare the geometry of the capillary network in different striated muscles (and species), in search of general concepts underlying microvascular architecture in this tissue.

As stated by Aristotle, "Here and elsewhere we shall not obtain the best insight into things until we see them growing from the beginning...." This study presents only a small fraction of the data required for understanding the biophysics of the microcirculation in skeletal muscle, but hopefully, it does provide a beginning.

VI. PROPOSALS FOR FUTURE RESEARCH

A requisite for understanding oxygen transport to tissue is a knowledge of the architecture of the capillary network, and an insight into the factors at work in its development. The cylindrical symmetry of the capillaries and fibres makes this tissue an excellent experimental model. This study was undertaken to improve quantitative methods in this field, and to begin the collection of the necessary information for understanding the principles underlying microvascular architecture in this tissue. A number of possibilities are suggested for future research by the results of this work. These can be roughly divided into two groups: (a) architectural studies and (b) developmental studies.

(a) The analysis of the capillary supply as seen in transverse sections of skeletal muscle suggests that the results may be representative of a wider range of animals, including humans. Using the more reliable PAS-Luxol Fast Blue technique, this hypothesis should be tested on a more diverse sample of both animals and muscles. Inclusion of distinctly "red" and distinctly "white" muscles, or identification of muscle fibre type by histochemical demonstration would settle the long-standing controversy over the vascular supply of "red and white" muscle.

Similarly the analysis of the branching pattern of the capillary network must be investigated in a number of muscles to ascertain the characteristics which are common in the architectural design of the network. Such a study would involve either stereological reconstruction of the vascular bed from many sections of a thick muscle, or preparation of corrosion-casted reproductions of the network using Batson's #17 Anatomical Corrosion Compound.

(b) Although the development of the capillary supply of cardiac muscle fibres has been studied by Hort (1955, 1970), no definitive study of this kind has yet been done for developing skeletal muscle. Studying the relationship of the network to the fibres during growth is essential to understanding the factors governing the development of the vascular supply. Slight alterations in the microenvironment, such as may occur with hypoxia, hyperoxia, exercise, etc., may promote the development of a radically different capillary bed, and should be carefully investigated.

APPENDIX I: Values of capillary and segment length in striated muscle reported in the literature*

Author (year)	Animal, muscle	Capillary length (mm) mean	Capillary length (mm) range	Segment length (mm) mean	Segment length (mm) range	Number loops per length	Methods employed
Rous et al. (1930)	rabbit adductor magnus	0.69	0.43-1.35	-	-	-	direct viewing of injected muscles
	rabbit external oblique	-	0.75-1.50	-	-	-	
Smith & Rous (1931)	chicken pectoralis major	-	0.33	-	-	-	same as Rous et al. (1930)
	frog sartorius	-	1.0 -2.0	-	-	-	
	pigeon pectoralis major	-	0.20	-	-	-	
	rabbit diaphragm	-	0.50	-	-	-	
	rabbit gracilis	-	1.0	-	-	-	
Steudel (1938)	frog sartorius	1.16[c]	0.52-2.31	0.56	0.14-1.28	2.09[c]	amyl nitrite vaso-dilation,
	frog gastrocnemius	1.19[c]	0.45-2.22	-	-	-	India ink injection,
	frog fascia latus	1.02[c]	0.51-2.07	-	-	-	fixation in 10%
	frog submaxillaris	0.72[c]	0.25-1.25	0.31	0.08-0.85	2.33[c]	formalin, embedded
	toad sartorius	1.10[c]	0.51-2.06	-	-	-	in gelatin, frozen
	salamder dorsalis trunci	1.58[c]	0.73-2.60	-	-	-	sections, ?
	salamder supracoideus	1.40[c]	0.77-2.46	-	-	-	
Nikiforova & Shoskenko (1963)	frog sartorius	-	0.25-1.00	-	<0.20	1-5[c]	direct observations
Grant and Wright (1964)	rat cremaster	-	1.0-2.0	-	-	-	direct observations
Markizow (1968)	human muscle	-	0.60-0.70	-	-	-	India ink, ?
Honig et al. (1970)	dog and rat gracilis	-	0.40-0.50	0.10	-	4-5[c]	direct observations
Smaje et al. (1970)	rat cremaster	0.62	-	0.21	-	2.95[c]	India ink, 10% formalin, glycerol clearing
Hammersen (1970)	rabbit adductor magnus	-	0.30-0.50	-	-	-	coloured gelatin, 10%
	rabbit rectus abdominis	-	0.75-1.30	-	-	-	formalin, cleared, gelatin embedded, frozen sections

Eriksson & Myrhage (1972)	cat tenuissimus	1.02	-	0.20	-	5.08[c]	papaverine, India ink, glutaraldehyde, formalin, clearing, ATPase activity
Nikolov & Schumacher (1972)	dog jaw muscles	-	-	-	0.18-0.22	-	India ink + gelatin, 10% neutral formalin, clearing, paraffin sections
Nikolov & Schumacher (1973)	dog tongue	-	0.12-0.15	-	0.02-0.03	5-10[c]	same as Nikolov and Schumacher (1972)
Eriksson et al. (1973)	cat tenuissimus	1.00	-	0.20	-	5	same as Eriksson and Myrhage (1972)
Ivanova (1973)	fish lateralis superior	0.62	-	-	-	-	India ink, 10% formalin, frozen sections
	fish lateralis magnus	1.05	-	-	-	-	
	frog rectus abdominis	1.12	-	-	-	-	
	frog sartorius	1.22	-	-	-	-	
	frog gastrocnemius	1.27	-	-	-	-	
	frog femoral tibialis	1.29	-	-	-	-	
	reptiles[a] sartorius	0.70[c]	-	-	-	-	
	reptiles gastrocnemius	0.69[c]	-	-	-	-	
	reptiles femoral tibialis	0.68[c]	-	-	-	-	
	reptiles semitendinosus	0.73[c]	-	-	-	-	
	birds[b] sartorius	0.54[c]	-	-	-	-	
	birds gastrocnemius	0.53[c]	-	-	-	-	
	birds soleus	0.53	-	-	-	-	
	rat masseter	0.49	-	-	-	-	
	rat gastrocnemius	0.52	-	-	-	-	
	rat tibialis anterior	0.51	-	-	-	-	
	rat semitendinosus	0.54	-	-	-	-	
	mouse masseter	0.32	-	-	-	-	
	mouse gastrocnemius	0.40	-	-	-	-	
	mouse tibialis anterior	0.36	-	-	-	-	
	mouse semitendinosus	0.36	-	-	-	-	
	dog gastrocnemius	0.55	-	-	-	-	
	dog soleus	0.55	-	-	-	-	
	dog tibialis anterior	0.55	-	-	-	-	
	dog semitendinosus	0.57	-	-	-	-	
	dog gracilis	0.56	-	-	-	-	
	squirrel gastrocnemius	0.34	-	-	-	-	
	squirrel soleus	0.34	-	-	-	-	
	squirrel semitendinosus	0.36	-	-	-	-	
	squirrel tibialis anterior	0.37	-	-	-	-	

Author (year)	Animal, muscle	Capillary length (mm) mean	Capillary length (mm) range	Segment length (mm) mean	Segment length (mm) range	Number loops per length	Methods employed
Sabaev (1973)	human muscle	-	0.40-3.40[p]	-	-	-	India ink, 5% formalin, ?
	human muscle	-	0.50-3.50[q]	-	-	-	
	human muscle	-	0.40-3.00[r]	-	-	-	
	human muscle	-	0.30-0.50[s]	-	-	-	
	human muscle	-	0.30-0.50[t]	-	-	-	
Mankovska (1973)	dolphin spinalis	0.42	-	-	-	-	?
	dolphin iliocostalis lateralis	0.42	-	-	-	-	
Hudlicka & Myrhage (1976)	rat extensor hallucis proprius	0.54[x]	-	-	-	-	?
	rat extensor hallucis proprius	0.51[y]	-	-	-	-	?
	rat extensor hallucis proprius	0.48[z]	-	-	-	-	
	rabbit tenuissimus	1.12[x]	-	-	-	-	
	rabbit tenuissimus	0.85[y]	-	-	-	-	
	rabbit tenuissimus	0.86[z]	-	-	-	-	

* guide to suprascripts

a - lizard and turtle
b - dove and chicken
c - calculated from data presented in the paper
p - 15 to 25 years old
q - 30 to 60 years old
r - 60 to 90 years old
s - newborn
t - foeti of 3 to 9 months
x - stimulation controls
y - stimulation for 7 days
z - stimulation for 14 days

APPENDIX II: Values of capillary supply of skeletal muscle fibres reported in the literature*

Author (year)	Animal, muscle	Cap. density	Fibre density	C/F ratio	N_{VF}	Other measures	Summary of method
Krogh (1919a)	horse gastrocnemius	1350[r]	-	-	-	-	India ink in gelatin injection, fixation in 5% formalin, paraffin embedding, no stain or eosin counter-staining
	dog semitendinosus	2630[r]	-	-	-	-	
	guinea pig masseter	2840[r]	-	-	-	-	
	guinea pig prae-tracheal	3700[r]	-	-	-	-	
	cod eye muscle	400[r]	-	-	-	-	
	frog sartorius	400[r]	-	-	-	-	
Krogh (1919b)	frog sartorius	322[cu]	-	-	-	-	same method as used in 1919a
	frog rectus abdominis	150[cu]	-	-	-	-	
	frog rectus abdominis	5[r]	-	-	-	-	
	guinea pig diaphragm	2567[cr]	-	-	-	-	
Stoel (1925)	rabbit adductor magnus	1550[r]	2130	0.73[c]	-	-	India ink, 10% formalin, celloidin, stained with H and van Gieson
	rabbit semitendinosus	790[r]	650	1.22[c]			
Duyff & Bouman (1927)	rabbit soleus	1695[r]	774	2.19[c]			India ink, 10% formalin, celloidin, stained with H and van Gieson
	rabbit medial gastrocnemius	1352[r]	874	1.56[c]			
	rabbit lateral gastrocnemius	1335[r]	782	1.71[c]			
	rabbit adductor magnus	-	1024	-			
	rabbit semitendinosus	1198[r]	391	3.06[c]			
	rabbit ancon. longus	1679[r]	1008	1.67[c]			
	rabbit biceps brachialis	1715[r]	1141	1.50[c]			
	rabbit biceps femoris	1065[r]	716	1.49[c]			
	rabbit rectus femoris	1465[r]	1241	1.18[c]			
Vannotti & Magiday (1934)	rabbit extensor	-	-	1.00[e]	-	-	formalin, ?, red cell staining
	rabbit adductor	-	-	1.03[e]			
	rabbit gluteus	-	-	0.92[e]	-	-	

Sjöstrand (1935)	mouse gastrocnemius	3022[cr]	-	-	-	-	20% neutral formalin + ferri-cyanide, paraffin, stained with orthotolidine + Erlich's H. for red cells
	mouse masseter	4393[cr]	-	-	-	-	
	guinea pig gastroc.	1136[cr]	-	-	-	-	
	guinea pig masseter	1927[cr]	-	-	-	-	
	mouse gastrocnemius	3575[ce]	-	-	-	-	
	mouse masseter	5100[ce]	-	-	-	-	
	guinea pig gastroc.	1917[ce]	-	-	-	-	
	guinea pig masseter	3117[ce]	-	-	-	-	
Petrén (1935)	guinea pig gastroc.	875[r]	-	-	-	-	same as Sjöstrand (1935)
	guinea pig masseter	1750[r]	-	-	-	-	
	guinea pig gastroc.	1500[e]	-	-	-	-	
	guinea pig masseter	1750[e]	-	-	-	-	
Lindgren (1935)	guinea pig gastroc.	1200[r]	-	-	-	-	H & E or method of Sjöstrand (1935)
	guinea pig pectoralis major	1270[r]	-	-	-	-	
Vannotti and Gukelberger (1935)	guinea pig surface layers	-	-	0.77[cr]	-	-	10% formalin, stained with Berlin blue
	guinea pig deep red layers	-	-	1.49[cr]	-	-	
	guinea pig surface layers	-	-	0.90[ch]	-	-	
	guinea pig deep red layers	-	-	1.71[ch]	-	-	
Petrén et al. (1936)	guinea pig gastroc.	1378[r]	684	2.02[c]	-	-	20% formalin, same as Sjöstrand (1935)
	guinea pig masseter	2873[r]	939	3.06[c]	-	-	
	guinea pig gastroc.	1750[e]	716	2.44[c]	-	-	
	guinea pig masseter	3026[e]	994	3.05[c]	-	-	
	" " vastus lateralis	2734[r]	1224	2.23[c]	-	-	
	guinea pig cruralis	1182[r]	874	1.35[c]	-	-	
	" " semimembranosus	1183[r]	799	1.48[c]	-	-	
	guinea pig extensor carpi radialis	1326[r]	849	1.56[c]	-	-	
	guinea pig flexor digitorum	1861[r]	974	1.91[c]	-	-	
Jores (1928)	rabbit adductor	-	-	0.6-1.4[r]	-	-	formalin, gelatin, ?
Perry (1930)	mouse anterior	1402[r]	-	-	-	-	?
	guinea pig anterior	1565[r]	-	-	-	-	

Author (year)	Animal, muscle	Cap. density	Fibre density	C/F ratio	N_{VF}	Other measures	Summary of method
Paff (1930)	rat rectus femoris	3573[r]	-	-	-	-	India ink, 10% formalin, paraffin, stained with Orange G
	rat tibialis anterior	3402[r]	-	-	-	-	
	quinea pig rectus femoris	3060[r]	-	-	-	-	
	quinea pig tibialis anterior	3501[r]	-	-	-	-	
	guinea pig gastrocnemius	2614[r]	-	-	-	-	
	cat rectus femoris	2474[r]	-	-	-	-	
	cat tibialis anterior	2214[r]	-	-	-	-	
	cat gastrocnemius	2341[r]	-	-	-	-	
Martin et al. (1932)	dog gracilis	1056[r]	1640	0.64	-	-	India ink, Bouin's fluid, paraffin, no stain or H & E
	dog gracilis	2010[e]	1640	1.23	-	-	
	dog gracilis	2580[v]	1291	2.00	-	-	
	puppy gracilis	1690[r]	2298	0.74	-	-	
	dog semimembranosus	3240[r]	4378	0.74	-	-	
	dog semimembranosus	5900[v]	3806	1.55	-	-	
Eriksson & Petrén (1937)	quinea pig masseter	2880[cr]	-	-	-	-	20% formalin, method of Sjöstrand (1935)
	guinea pig masseter	2920[ct]	-	-	-	-	
	quinea pig diaphragm	2928[ct]	-	-	-	-	
	quinea pig psoas	2423[ct]	-	-	-	-	
	quinea pig adductor	2290[ct]	-	-	-	-	
	quinea pig masseter	2730[cw]	-	-	-	-	
	quinea pig diaphragm	2360[cw]	-	-	-	-	
	quinea pig psoas	1975[cw]	-	-	-	-	
	quinea pig adductor	1873[cw]	-	-	-	-	
Sylvén (1938)	quinea pig gastrocnemius	1400[r]	-	-	-	-	20% formalin, method of Sjöstrand (1935)
	quinea pig masseter	2890[r]	-	-	-	-	
	guinea pig gastroc.	2330[e]	-	-	-	-	
	guinea pig masseter	3150[e]	-	-	-	-	
Steudel (1938)	frog sartorius	368[cr]	241	1.53	-	-	India ink, 20% formalin, gelatin or freezing, ?
	frog triceps femoris	356[cr]	361	0.99	-	-	
	frog gastrocnemius	331[cr]	140	2.36	-	-	
	toad sartorius	474[cr]	538	0.88	-	-	
	toad triceps femoris	527[cr]	439	1.20	-	-	
	toad gastrocnemius	400[cr]	440	0.91	-	-	
Gorkiewicz (1948)	trout parietal	[illegible]3[r]	-	-	-	-	?
	toad rectus lateralis	400[r]	-	-	-	-	
	toad eye muscle	276[r]	-	-	-	-	

Bösiger (1950)	hen breast muscle	-	-	-	1[r]	-	paraffin, stained with Heidenhain H, Light Green, Azure B or picro-indigo carmen for red cells
	starling breast muscle	-	-	-	4-6[r]	-	
	quail breast muscle	-	-	-	0-5[r]	-	
Clark et al. (1953)	tibialis anticus	-	-	-	-	39% increase over resting value[a]	?
Becker et al. (1955)	puppy gastrocnemius	-	-	-	-	area ratio 4.7%[r]	method of Lepchne or H & E for red cells
	puppy gasrrocnemius	-	-	-	-	area ratio 11.6%[a]	
Smith and Giovacchini (1956)	rabbit gracilis	-	-	0.50[r]	-	-	India ink, formalin, paraffin, counter-stained with eosin
	rabbit semitendinosus	-	-	1.70[r]	-	-	
Valdivia (1956)	guinea pig rectus anterior	1659[r]	638	2.64	6.8	-	India ink, 10% formalin, paraffin after gelatin, counter-atained with eosin
	guinea pig vastus exterior	1796[r]	642	2.87	7.4	-	
	guinea pig lateral gastrocnemius	1449[r]	579	2.51	-	-	
	quinea pig medial gastrocnemius	1493[r]	580	2.60	-	-	
	guinea pig rectus anterior	2326[a]	679	3.45	8.1	-	
	quinea pig vastus exterior	2407[a]	631	3.84	9.1	-	
	quinea pig lateral gastrocnemius	2103[a]	617	3.44	-	-	
	guinea pig medial gastrocnemius	2207[a]	628	3.52	-	-	
Heroux and St. Pierre (1957)	rat soleus	454[x]	295	1.60	-	-	5% neutral formalin, alcohol to toluol to paraffin, stained with Benzidine and Aniline Blue
	rat plantaris	125[x]	387	0.30	-	-	
	rat lateral gastrocnemius	435[x]	395	1.10	-	-	
	rat white areas of gastrocnemius	87[x]	-	-	-	-	
	rat soleus	594[y]	422	1.40	-	-	

Author (year)	Animal, muscle	Cap. density	Fibre density	C/F ratio	N_{VF}	Other measures	Summary of method
Valdivia (1958)	guinea pig white areas vastus lateralis	746^{pf}	540	1.37	-	-	India ink, 10% formalin, same as Valdivia (1956)
	guinea pig white thigh muscle	686^{pf}	460	1.48	-	-	
	guinea pig red vastus lateralis	1897^{pf}	630	3.01	7.4	-	
	guinea pig red vastus lateralis	1682^{p}	565	2.96	7.8	-	
	guinea pig red vastus lateralis	2407^{a}	631	3.84	9.1	-	
	guinea pig red areas gastrocnemius	1582^{cpf}	638	2.50	-	-	
	guinea pig red areas gastrocnemius	1488^{cp}	599	2.48	6.9	-	
	guinea pig red areas gastrocnemius	2155^{ca}	623	3.48	-	-	
	guinea pig red areas rectus femoris	1702^{pf}	633	2.71	6.8	-	
	guinea pig red areas rectus femoris	1620^{p}	694	2.33	7.2	-	
	guinea pig red areas rectus femoris	2326^{a}	679	3.45	8.1	-	
Lobyntsev (1959)	mouse soleus	2900^{r}	-	-	-	-	India ink, 10% formalin, celloidin-paraffin, counter-stained with eosin
	mouse soleus	3920^{s}	-	-	-	-	
	mouse soleus	2385^{d}	-	-	-	-	
	mouse vastus lateralis	2100^{r}	-	-	-	-	
	" " "	3745^{s}	-	-	-	-	
	" " "	3223^{d}	-	-	-	-	
Valdivia et al. (1960)	guinea pig white areas rectus femoris	-	-	-	1.0^{r}	-	Helly's fluid, paraffin, H & E or acid fuchsin for red cells
	guinea pig white areas rectus femoris	-	-	-	1.0^{m}		
	guinea pig white areas rectus femoris	-	-	-	1.0^{n}	-	
	guinea pig vastus lateralis	-	-	-	1.0^{r}	-	
	guinea pig vastus lateralis	-	-	-	1.0^{m}	-	
	guinea pig vastus lateralis	-	-	-	1.0^{n}	-	
	guinea pig red areas rectus femoris	-	-	-	2.4^{r}	-	
	" " " "	-	-	-	3.95^{m}	-	
	" " " "	-	-	-	4.3^{n}	-	
	guinea pig vastus lateralis	-	-	-	2.4^{r}	-	
	" "	-	-	-	3.95^{m}	-	
	" "	-	-	-	4.3^{n}	-	

Schmidt-Nielsen & Pennycuik (1961)	bat masseter	3207[r]	2018	1.6	-	-	India ink, 10% formalin, paraffin, counter-stained with eosin
	mouse masseter	2272[r]	1280	1.8	-	-	
	rat masseter	12 0[r]	713	1.7	-	-	
	guinea pig masseter	1704[r]	638	2.7	-	-	
	cat masseter	760[r]	843	0.9	-	-	
	rabbit masseter	1049[r]	495	2.1	-	-	
	dog masseter	830[r]	379	2.2	-	-	
	sheep masseter	1427[r]	688	2.1	-	-	
	pig masseter	695[r]	341	2.0	-	-	
	cattle masseter	956[r]	390	2.5	-	-	
	bat gastrocnemius	2224[r]	2473	0.9	-	-	
	mouse gastrocnemius	975[cr]	761	1.28	-	-	
	rat gastrocnemius	664[cr]	387	1.72	-	-	
	guinea pig gastroc.	1052[cr]	436	2.41	-	-	
	cat gastrocnemius	379[cr]	515	0.74	-	-	
	rabbit gastrocnemius	294[cr]	268	1.10	-	-	
	dog gastrocnemius	782[cr]	285	2.74	-	-	
	sheep gastrocnemius	339[r]	293	1.16	-	-	
	pig gastrocnemius	339[r]	305	1.11	-	-	
	cattle gastrocnemius	304[r]	307	0.99	-	-	
Nikiforova & Shoshenko (1963)	frog sartorius	60-150	-	-	-	-	direct viewing
Henneman & Olson (1964)	cat A fibres	-	-	-	0[r]	-	frozen sections stained for ATPase activity
	B fibres	-	-	-	4-8[r]	-	
	C fibres	-	-	-	4-8[r]	-	
	from gastrocnemius and soleus						
Romanul (1965)	rat, rabbit						
	A fibres	-	-	-	1-2[r]	-	frozen sections stained for alkaline phosphot-ase activity or India ink
	B fibres	-	-	-	6-8[r]	-	
	C fibres	-	-	-	6-8[r]	-	
	from gastrocnemius, soleus & plantaris						
Nishiyama (1965)	rat, cat						
	A fibres	-	-	-	1-2[r]	-	frozen sections, stained for succinic dehydro-genase activity and injected with India ink
	B fibres	-	-	-	3-4[r]	-	
	C fibres	-	-	-	4-6[r]	-	
	from gastrocnemius and soleus						

Author (year)	Animal, muscle	Cap. density	Fibre density	C/F ratio	N_{VF}	Other measures	Summary of method
Shoshenko (1966)	rat gastrocnemius	-	-	-	-	area ratio 5.8%^{r}	?
	rat extensor digitorum longus	-	-	-	-	area ratio 5.9%^{r}	
	rat quadriceps femoris	-	-	-	-	area ratio 6.2%^{r}	
	rat tibialis posterior	-	-	-	-	area ratio 6.6%^{r}	
	rat soleus	-	-	-	-	area ratio 6.7%^{r}	
	rat peroneus	-	-	-	-	area ratio 6.7%^{r}	
	rat adductor magnus	-	-	-	-	area ratio 6.7%^{r}	
	rat prae-brachialis	-	-	-	-	area ratio 6.7%^{r}	
	rat semimembrancsus	-	-	-	-	area ratio 7.0%^{r}	
	rat biceps femoris	-	-	-	-	area ratio 6.8%^{r}	
	rat adductor brevis	-	-	-	-	area ratio 6.9%^{r}	
	rat tibialis anterior	-	-	-	-	area ratio 6.9%^{r}	
	rat gluteus medialis	-	-	-	-	area ratio 7.2%^{r}	
	rat semitendinosus	-	-	-	-	area ratio 7.2%^{r}	
	rat adductor longus	-	-	-	-	area ratio 7.3%^{r}	
	rat rectus abdominis	-	-	-	-	area ratio 7.7%^{r}	
Czopek and Czopek (1967)	amphibian arm muscle	251^{cr}	301	0.83	-	-	Prussian blue, 10% formalin, ?
	amphibian forearm muscle	247^{cr}	293	0.84	-	-	
	amphibian thigh muscle	238^{cr}	298	0.80	-	-	
	amphibian lower leg muscle	238^{cr}	294	0.81	-	-	
	amphibian submaxillaris	209^{cr}	436	0.48	-	-	
	amphibian rectus abdominis	109^{cr}	208	0.52	-	-	
	amphibian longissimus dorsi	110^{cr}	209	0.52	-	-	
	amphibian tail muscle	93^{cr}	208	0.44	-	-	
Stulcova & Hudlicka (1967)	rat soleus	-	-	1.65^{r}	-	-	India ink, Bouin's fluid, paraffin, stained with picro-fuchsin
	rat soleus	-	-	1.73^{o}	-	-	
	rat soleus	-	-	1.47^{z}	-	-	
Rakusan and du Mensil de Rochemont (1967)	dog gastrocnemius	-	-	-	-	volume ratio 0.64%^{v}	frozen, minced tissue, counted radioactivity (I^{131})
	dog vastus lateralis	-	-	-	-	" 0.39%^{v}	
	rabbit gastrocnemius	-	-	-	-	" 1.29%^{v}	
	rabbit vastus lateralis	-	-	-	-	" 1.07%^{v}	

Carrow et al. (1967)	rat red fibres	-	-	2.53^{r}	-	-	India ink, 10% formalin, gelatin, frozen sections stained for ATPase activity
	rat red fibres	-	-	2.65^{pf}	-	-	
	rat red fibres	-	-	2.63^{d}	-	-	
	rat white fibres	-	-	1.22^{r}	-	-	
	rat white fibres	-	-	1.42^{pf}	-	-	
	rat white fibres	-	-	1.35^{d}	-	-	
Saltin et al. (1968)	human quadriceps	197^{cr}	-	-	-	-	electron microscopy
	human quadriceps	200^{cb}	-	-	-	-	
	human quadriceps	226^{cd}	-	-	-	-	
Cooper et al. (1969)	pig longissimus	-	-	0.33^{r}	-	-	frozen sections stained for alkaline phosphotase activity by the azo dye technique
	pig trapezius	-	-	1.20^{r}	-	-	
Romanul and Pollock (1969)	rat soleus^{ri}	-	-	-	-	fibres with low succinic dehydrogenase activity had few capillaries while those with high activity had many. Thyroxine administration led to an increased capillary density in all muscles	frozen sections stained for alkaline phosphotase activity
	rat flexor digitorum longus^{ri}	-	-	-	-		
	rat flexor hallucis longus^{ri}	-	-	-	-		
	rat gastrocnemius^{r}	-	-	-	-		
	rat plantaris^{r}	-	-	-	-		
	rat soleus^{r}	-	-	-	-		
	rat gastrocnemius^{t}	-	-	-	-		
	rat plantaris^{t}	-	-	-	-		
	rat soleus^{t}	-	-	-	-		
Smaje et al. (1970)	rat cremaster	1300	-	-	-	-	India ink, 10% formalin, glycerol clearing, ?
Mai et al. (1970)	guinea pig soleus and gastrocnemius						
	white fibres	-	-	-	3.9^{r}	-	frozen sections stained for alkaline phosphotase activity
	white fibres	-	-	-	3.8^{d}	-	
	red fibres	-	-	-	4.5^{r}	-	
	red fibres	-	-	-	5.8^{d}	-	
	modified red fibres	-	-	-	4.6^{r}	-	
	modified red fibres	-	-	-	4.9^{d}		
	intermediate fibres	-	-	-	5.3^{r}	-	
	intermediate fibres	-	-	-	5.2^{r}	-	

Author (year)	Animal, muscle	Cap. density	Fibre density	C/F ratio	N_{VF}	Other measures	Summary of method
Hammersen (1970)	rabbit sartorius	300[r]	522	0.57	-	-	Berlin blue + gelatin,
	rabbit gracilis	219[r]	321	0.68	-	-	10% formalin, gelatin,
	rabbit adductor magnus	244[r]	383	0.64	-	-	frozen sections,
	rabbit semitendinosus	247[r]	210	1.18	-	-	Mayer's H
	cat sartorius	281[r]	150	1.87	-	-	
	cat rectus femoris	811[r]	778	1.04	-	-	
Miller & Hale (1970)	rat gastrocnemius	790[r]	-	-	-	-	India ink, formalin,
	rat gastrocnemius	1159[a]	-	-	-	-	paraffin, counter-
	rat gastrocnemius	1113[j]	-	-	-	-	stained with eosin
	rat gastrocnemius	887[l]	-	-	-	-	
	rat gastrocnemius	1228[k]	-	-	-	-	
Honig et al. (1970)	rat gracilis	240[r]	-	-	-	-	direct observations
		624[m]	-	-	-	-	
Hermansen & Wachtlova (1971)	human quadriceps	640[g]	440	1.49	-	-	10% formalin, PAS
	human quadriceps	611[gd]	414	1.57	-	-	method of Hecht
	human quadriceps	600[q]	557	1.08	-	-	
	human quadriceps	599[qd]	576	1.05	-	-	
Parizkova et al. (1971)	human quadriceps	270[yq]	329	0.81	-	-	10% formalin, PAS
	human quadriceps	362[Og]	556	0.65	-	-	method of Hecht
	human quadriceps	315[Oq]	533	0.59	-	-	
Cassin et al. (1971)	rat gracilis	109[r]	-	-	-	-	absolute alcohol at
	rat gracilis	154[a]	-	-	-	-	4°C, benzidine clearing,
	rat gracilis	-	-	0.91[r]	-	-	paraffin, stained for
	rat gracilis	-	-	0.89[a]	-	-	alkaline phosphotase
	rat plantaris	-	-	1.80[r]	-	-	activity + Mayer's H
	rat plantaris	-	-	1.78[a]	-	-	
Romanul (1971)	see Romanul & Pollock (1969)	-	-	-	-	same conclusions as R & P (1969)	same method as R & P (1969)
Schmalbruch (1971)	rat tibialis anterior	-	-	1.60[cr]	-	-	calcium-formol, epon
	rat soleus	-	-	2.80[r]	-	-	embedded, stained with
	quinea pig gastrocnemius	-	-	1.20[r]	-	-	toluidine blue
	guinea pig soleus	-	-	2.60[r]	-	-	
	guinea pig vocalis	-	-	0.70[r]	-	-	
	guinea pig thyreoaryt.	-	-	0.70[r]	-	-	
	cat gastrocnemius	-	-	1.00[r]	-	-	
	cat soleus	-	-	1.90[r]	-	-	

Asmussen et al. (1971)	cat and guinea pig extra-ocular muscles	1900^{rA} 700^{rB}	1580 875	1.2 0.8	- -	- -	frozen sections stained for ATPase activity
Rakusan et al. (1971)	pigeon breast muscle pigeon breast muscle	2075^{cf} 2429^{cp}	1646 1967	1.27 1.23	- -	- -	neutral formalin, ?, stained with PAS + van Gieson
Watchlova and Parizkova (1972)	rat gastrocnemius rat gastrocnemius rat gastrocnemius rat soleus rat soleus rat soleus hare gastrocnemius hare soleus rabbit gastrocnemius rabbit soleus	1577^{Wr} 1780^{Lr} 1618^{er} 1948^{Wr} 1265^{Lr} 1693^{er} 1682^{r} 1509^{r} 661^{r} 743^{r}	1317 930 883 1519 670 880 845 754 518 466	1.22 1.92 1.85 1.32 1.89 1.72 1.96 2.00 1.28 1.56	- - - - - - - - - -	- - - - - - - - - -	10% formalin, PAS method of Hecht
Eriksson and Myrhage (1972)	cat tenuissimus A fibres B fibres C fibres overall	- - - -	- - - -	- - - 0.95^{r}	3.5^{r} 3.6^{r} 3.8^{r} 3.6^{r}	- - - -	India ink, glutaraldehyde, formalin, ethanol, 85% benzol-benzoate, oil of Wintergreen
Nikolov and Schumacher (1972)	dog masseter dog temporalis dog pteryoid.	- - -	- - -	$3.6\text{-}4.0^{cr}$ $2.8\text{-}3.2^{cr}$ 3.0^{cr}	- - -	- - -	India ink + gelatin, 10% formalin, paraffin, H & E or van Gieson
Cotter et al. (1973)	rabbit exten. digit. longus " " " " " " " " "	530^{r} 540^{r} 690^{F} 1200^{G}	424 383 448 550	1.25 1.41 1.54 2.18	- - - -	- - - -	?, alkaline phosphotase activity
Taylor et al. (1973)	cat jaw muscles	-	-	-	3-4	-	frozen sections, sta stained for ATPase activity or by PAS
Mankovska (1973)	dolphin spinalis dolphin iliocostalis lateralis	1014^{r} 2210^{r}	- -	- -	- -	- -	?
Eriksson et al. (1973)	cat tenuissimus	1300^{r}	1350	0.95	-	-	same as Eriksson and Myrhage (1972)

Author (year)	Animal, muscle	Cap. density	Fibre density	C/F ratio	N_{VF}	Other measures	Summary of method
Ivanova (1973)	fish lateralis superior	683^{cr}	456	1.50	-	-	10% formalin, frozen sections, ?
	fish lateralis magnus	93^{cr}	119	0.78	-	-	
	frog sartorius	75^{cr}	78	0.96	-	-	
	frog gastrocnemius	65^{cr}	67	0.97	-	-	
	reptiles trapezius	617^{cr}	437	1.41	-	-	
	reptiles biceps brachialis	707^{cr}	591	1.20	-	-	
	reptiles sartorius	583^{cr}	431	1.35	-	-	
	reptiles gastrocnemius	612^{cr}	468	1.31	-	-	
	reptiles semitendinosus	593^{cr}	481	1.23	-	-	
	dove sartorius	1150^{r}	838	1.37	-	-	
	dove gastrocnemius	970^{r}	612	1.59	-	-	
	dove soleus	1140^{r}	766	1.49	-	-	
	chicken sartorius	1700^{r}	478	3.56	-	-	
	chicken gastrocnemius	2400^{r}	658	3.65	-	-	
	chicken soleus	2390^{r}	701	3.41	-	-	
	rat masseter	1090^{r}	846	1.29	-	-	
	rat gastrocnemius	920^{r}	553	1.68	-	-	
	rat tibialis anterior	1070^{r}	930	1.15	-	-	
	rat semitendinosus	920^{r}	450	2.04	-	-	
	mouse masseter	1400^{r}	961	1.47	-	-	
	mouse gastrocnemius	1000^{r}	567	1.76	-	-	
	mouse tibialis anterior	1030^{r}	658	1.55	-	-	
	mouse semitendinosus	1025^{r}	623	1.73	-	-	
	dog gracilis	1730^{r}	1155	1.50	-	-	
	dog semitendinosus	1900^{r}	982	1.94	-	-	
	dog gastrocnemius	1715^{r}	612	2.80	-	-	
	dog soleus	2010^{r}	982	2.05	-	-	
	dog tibialis anterior	2400^{r}	1291	1.87	-	-	
Lie (1974)	lamprey parietal fibres	-	-	-	-	$1/100\ \mu m^{N}$	frozen sections, ?
	lamprey next to parietal fibres	-	-	-	-	$1/147\ \mu m^{N}$	
Czopek (1974)	salamander thigh	222^{cr}	346	0.64	-	-	Prussian blue, 10% formalin, ?
	salamander lower leg	210^{cr}	332	0.63	-	-	
	salamander submaxillaris	283^{cr}	540	0.52	-	-	
	" rectus abdominis	178^{cr}	329	0.54	-	-	
	" longissimus dorsi	122^{cr}	317	0.38	-	-	
	" tail muscle	118^{cr}	248	0.47	-	-	
Jerusalem et al. (1974)	human children	-	-	-	-	951^{rz}	?, epon embedded, electron microscope
	limb muscles	-	-	-	-	930^{xz}	

Asmussen and Kiessling (1974)	lamprey mitochondrion-rich fibres	-	-	-	4-5[r]	-	frozen sections, ?
	lamprey mitochondrion-poor fibres	-	-	-	2[r]	-	
	lamprey tonus bundles	160[r]	-	-	-	-	
Eisenberg et al. (1974)	guinea pig soleus	-	-	-	-	area ratio 2.05%[r]	glutaraldehyde (buffered, pH 7.2), epon-embedded, electron microscope
Eisenberg and Kuda (1975)	guinea pig white vastus	-	-	-	-	area ratio 0.8%[r]	same as Eisenberg et al. (1974)
Casley-Smith et al. (1975)	dog gastrocnemius	1310[r]	-	-	-	-	glutaraldehyde, osmic tetroxide, acetone, araldite, lead citrate, uranyl acetate, transmission electron microscope
Banchero (1975)	dog sternothyroideus	624[r]	306	2.04	-	-	10% buffered formalin, paraffin, PAS method of Hecht
		1263[a]	610	2.07	-	-	
Melman et al. (1975)	human muscle	585[r]	-	-	-	-	?
Yamaki et al. (1975)	mouse cremaster	-	-	-	-	volume ratio 3.24%[r]	carbon injection
Andersen (1975)	human quadriceps	317[M]	190	1.67	-	-	frozen sections stained with amylase-PAS
		287[q]	220	1.30	-	-	
		355[qd]	165	2.15	-	-	
		356[qd]	183	1.95	-	-	
Myrhage and Hudlicka (1976)	rat extensor hallus longus	1347[A]	1333	1.01	-	-	frozen sections stained for alkaline actomyosin ATPase
		1043[B]	1122	0.93[M]	-	-	
		-	-	0.95	-	-	

Author (year)	Animal, muscle	Cap. density	Fibre density	C/F ratio	N_{VF}	Other measures	Summary of method
Prewitt and Johnson (1976)	rat cremaster	1045^{cP}	-	-	-	-	direct observation
		739^{cQ}	-	-	-	-	
		589^{cR}	-	-	-	-	
		283^{cS}	-	-	-	-	
Eby and Banchero (1976)	dog sternothyroideus	2016^{r}	1720	1.17	-	-	10% buffered formalin, paraffin, stained with PAS + van Gieson
Brown et al. (1976)	rabbit exten. digit. longus	-	-	1.27^{cr}	-	-	frozen sections stained for alkaline phosphotase activity by the method of Gomori
	" " "	-	-	1.54^{I}_{J}	-	-	
	" " "	-	-	1.74^{K}	-	-	
	" " "	-	-	2.18^{T}	-	-	
	" " "	-	-	1.73^{U}	-	-	
	" " "	-	-	1.73^{V}	-	-	
	rabbit flexor hallucis longus	-	-	1.28^{r}	-	-	
	" " "	-	-	1.14^{V}	-	-	
	rabbit soleus	-	-	1.16^{r}	-	-	
	rabbit soleus	-	-	2.1^{V}	-	-	
				2.2	-	-	

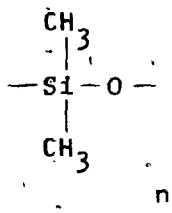
* guide to suprascripts

a - altitude
b - bed rest
c - calculated from data presented in paper
d - dynamic training
e - exercise
f - free to run in pens
g - well-trained
h - histamine injections
i - cross-innervation
j - cobalt chloride injections
k - transfusion
l - transfusion controls (sham)
m - hypoxic periods
n - chronic hypoxia
o - tenotomy
p - close confined in pens
q - untrained
r - resting (normal, control, etc.)
s - static training
t - thyroxin injections
u - stimulated electrically
v - vasodilated
w - thyroidectomy
x - animals kept at 30°C
y - animals kept at 6°C
z - sham operated tenotomy controls

A - proximal end
B - distal end
C - stimulated at 40 Hz
D - stimulated at 10 Hz
E - eosin
F - four days stimulation
G - 28 days stimulation
H - haematoxylin
I - continuous stimulation at 10 Hz for four days
J - continuous stimulation at 10 Hz for 14 days
K - continuous stimulation at 10 Hz for 28 days
L - laboratory
M - mean value
N - one vessel per length of circumference
O - old men
P - 0% oxygen
Q - 5% oxygen
R - 10% oxygen
S - 21% oxygen
T - continuous stimulation at 5 Hz for 14 days
U - intermittent stimulation at 10 Hz for 21 days
V - intermittent stimulation at 40 Hz for four days
W - wild
X - dystrophic muscle
Y - young men
Z - area of fibre supplied by one capillary

APPENDIX III: Properties of Microfil*

Microfil is a silicone elastomer, a non-vinyl polymer based on oxygen-silicone linkages with organic groups attached to the silicon atoms (Hey, 1966; Pearce, 1972).



The mixing procedure consists of adding a catalyst (5% by weight or volume) to equal weights of MV compound (coloured component) and MV diluent (clear component) or volumes in the ratio 4:5. The physical properties of the components are given in Table AIII-I. Curing takes place as a "non-exothermic crosslinking with minimal volume change" (manufacturer's description). Initial viscosity of the mixture is in the range of 15-25 cP and working time is approx. 15 minutes. Particle size (1-2.5 μm), a viscosity compatible with free flow of injectate at physiological pressure gradients, its immicibility with blood (and water) and a choice of colours for multiple injections makes Microfil an ideal compound for microvascular casting.

Several procedures should be employed to facilitate perfusion and to enhance resolution of vascular structure:

(1) use of a vasodilator for observing the total vascular bed

(2) heparinization to maintain blood fluidity

(3) washing out of the bed with saline prior to Microfil infusion

(4) deaeration (two minutes under vacuum) of the injection mass to remove air bubbles

(5) refrigeration of specimens while curing completes, eliminates autolysis

(6) two types of tissue clearing have been used:

(a) alcohol-methyl salicylate technique (see #7 for drawback)

(b) glycerine technique (see #7 for drawback)

(7) the use of organic compounds (especially alcohols) produces considerable volume change in the elastomer, making quantitative measurements more difficult.

In the studies discussed here, MV112 and MV122 compounds were used in filling the complete vascular bed of the muscles, and MV122 with MV High Viscosity Diluent (initial viscosity 450 cP) was used to demonstrate the arterial supply of frog sartorius in the longitudinal analysis.

*Microfil - product of Canton Biomedical Products, Inc., P. O. Box 2017, Boulder, Colorado, 80302.

Table AIII-I

Typical physical properties of Microfil compounds[1]

Property	Compound				
	MV112	MV117[2]	MV118	MV122[2]	MV Diluent
colour	white	orange	maroon	yellow	clear
specific gravity	1.28	1.32	1.27	1.30	0.96
viscosity (cP)[3]	120	200	120	160	5
non-volatile content (%)	64	64	64	64	-
get time (minutes)[4]	100	75	100	100	-
useful shelf life (months)	4	4	4	4	indefinite

1. data from Canton Biomedical Products, Inc.
2. MV112 and MV122 contain a lead chrome matrix which offers a high x-ray absorption
3. measured on a Brookfield model LVF viscometer with a number 2 spindle at 30 rpm.
4. gel time is the time required for the mixture to cease flowing.

APPENDIX IV: Composition of solutions

(1) 10% buffered formalin (buffered to pH 7.4)

NaH_2PO_4	2.7 gr
Na_2HPO_4	4.3 gr
HCHO (40%)	100.0 ml
H_2O (distilled)	900.0 ml

(2) 7% buffered formalin (buffered to pH 7.4)

NaH_2PO_4	2.7 gr
Na_2HPO_4	4.3 gr
HCHO (40%)	70.0 ml
H_2O (distilled)	930.0 ml

(3) frog Ringers solution[1]

NaCl	112.1 mM
KCl	1.9 mM
$CaCl_2$	0.8 mM
$NaHCO_3$	2.4 mM

1. from GENTRY and JOHNSON (1972).

APPENDIX V: Staining procedure for modified Gomori trichrome stain*

Fixation: 10% buffered formalin

Sectioning: paraffin sections, 3-15 µm

Solutions: trichrome stain:

Chromotrope 2R	0.6 gm
Light green, SF yellowish	0.3 gm
glacial acetic acid	1.0 ml
phosphotungstic acid	0.8 gm
distilled H_2O	100.0 ml

Staining procedure:

(1) deparaffinize and hydrate to distilled H_2O

(2) trichrome stain for 15 to 20 minutes

(3) place in 0.5% glacial acetic acid for two minutes.

Note: If sections are too dark, differentiate in 1% glacial acetic acid to which 0.7 gm of phosphotungstic acid has been added, then rinse in distilled H_2O.

(4) dehydrate in graded solutions of alcohol to absolute. Clear in xylene, and mount.

Results:

muscle fibres	-	blue-green
nuclei	-	very pale purple (if visible at all)
collagen	-	green
red cells	-	brilliant red

*Method modified by Z. J. Pattison, Dept. of Pathology, U.W.O. from that in Luna (1968), pp. 93-94.

APPENDIX VI: PAS-Luxol Fast Blue staining technique.

The difficulties encountered in obtaining consistent results with the PAS method of Hecht (1958) led to the development of a method based on the luxol Fast Blue-PAS technique of Margolis and Pickett (1956). In the past, this method has been used primarily in looking at nerve cells and fibres. Mrs. Z. J. Pattison, Dept. of Pathology, U. W. O., modified the technique for use with muscle tissues. This modified PAS-Luxol Fast Blue procedure is suitable for use with either frozen sections, or with sections from tissue fixed in formalin or formol alcohol, and embedded in either celloidin or paraffin. Unlike other histochemical procedures, commonly being used, this method is relatively simple yet appears to give very clear and uniform staining of capillaries throughout the whole section. It is currently being used by L. Willner in a "follow-up" project on the effect of perfusion pressure on capillary filling with Microfil.

The following is the text of a paper on this technique recently submitted to Microvascular Research.

INTRODUCTION

The number of the capillaries in skeletal muscle and their spatial distribution with respect to the individual muscle fibres are of fundamental importance for an understanding of the transport of O_2 and nutrients to tissue. Several methods have been used for the visualization of capillaries in histological sections of skeletal muscle, but all appear to be beset with problems. It is not difficult to demonstrate the presence of numerous capillaries in muscle, but it is exceedingly difficult to be certain that all the vessels in a given section have been rendered visible.

The first method to be described (Krogh, 1919) involved the injection of an opaque material into the vascular system. Basically, two types of material have been employed, India ink or other particulates in gelatin (Krogh, 1919), and silicone elastomers (Plyley & Groom, 1975). The injection method is however only as good as the degree of capillary filling obtained. Two mechanisms relating to the injectate are known to contribute to an underperfusion of the capillary bed: (i) obstruction of the vessel lumens as a result of aggregation of the particles in the injectate (Hartman, Evans & Walker, 1929) and, (ii) constriction of arteries and arterioles on infusion of India ink (Smaje, Zweifach & Intaglietta, 1970). Obviously the maximum degree of filling obtainable will depend on the degree of patency of the vascular bed. The result of

underperfusion due to any of the above possibilities, is that patchiness of filling is apparent upon examination of transverse histological sections. This patchiness manifests itself either uniformly over the whole cross-section (Willner, unpublished results) or as an underperfusion of some fraction of the fasciculi (Martin, Wooley, & Miller, 1932; Plyley & Groom, 1975).

To obtain the maximum degree of capillary filling possible, Krogh (1919) perfused the muscle with vasodilator drugs in saline, followed by close arterial injection of the medium under high pressure; whenever possible considerable back-pressure was produced by clamping the venous outflow. Following this method and using papaverine as the vasodilator, the degree of filling, based on an erythrocyte staining method, was found in our experiments to exceed 95% (Plyley & Groom, 1975). However, capillaries containing only plasma at the point of sectioning would not have been identified. This problem is also encountered in all studies using erythrocyte staining as a means of identifying capillaries (Sjöstrand, 1935 and his co-workers; Heroux & St. Pierre, 1957).

To overcome the inadequacies of both the injection and red cell staining methods, histochemical procedures have been developed, eg. alkaline phosphatase (Romanul, 1965; Cooper, Cassens & Briskey, 1969) and periodic acid-Schiff (Hort, 1955; Hecht, 1958), which stain selectively certain components of the capillary wall. However, with regard to the alkaline phosphatase method, bare spots or patches of no reaction have been found and the "stain intensity in the capillaries varied throughout each section" (Cooper, Cassens & Briskey, 1969). Using the PAS method as modified by Hecht (1958) we have found a similar patchiness of staining, even within a given fasciculus. This makes measurements of

capillary-fibre geometry on a fascicular basis (Plyley & Groom, 1975) difficult, if not impossible.

In the course of trying various modifications of the PAS method, we have produced a procedure which appears to give a very clear and uniform staining of capillaries over the whole muscle cross-section. This paper reports the method and results obtained.

MATERIALS AND METHODS

PAS-Luxol Fast Blue staining procedure

1. Slides taken down to water: via two changes of xylol; absolute alcohol; 95% alcohol, 70% alcohol; distilled water.
2. Place in 0.5% periodic acid for seven minutes.
3. Rinse in running tap-water for ten minutes*.
4. Rinse in two changes of distilled water.
5. Blot dry.
6. Place in Schiff's reagent for 20-30 minutes.
7. Rinse in running tap-water for ten minutes.
8. Place in 0.1% Luxol Fast Blue for five minutes**.
9. Blot dry, and place in 37°C oven for 20-30 minutes.
10. Place in two changes of xylol***.
11. Mount in DPX mountant.

NOTES:

* No reducing solution is used (see Culling pp. 228-229).

** No rinse is used after staining with Luxol Fast Blue.

*** No steps through alcohol are necessary.

Solutions:

1. 0.5% periodic acid:

   periodic acid 0.5 g; distilled water 100 ml.

2. Schiff's reagent of Barger and De Lamater (see Culling p.216):

   (i) 1 gr basic fuchsin is dissolved in 400 ml distilled water, using heat if necessary.

   (ii) add 1 ml thionyl chloride ($SOCl_2$), stopper flask and, after shaking, allow to stand for 12 hours.

   Caution: material highly toxic and corrosive; this step should be carried out in a fume hood.

   (iii) add two gr activated charcoal, shake and immediately filter. This solution will be stable for several months if stored in a well-stoppered dark bottle in the refrigerator.

3. 0.1% Luxol Fast Blue:

   Luxol Fast Blue 0.1 gr; 95% alcohol 100 ml.

Comment:

The above method is suitable for use either with frozen sections, or with sections from tissue fixed in formalin or formol alcohol and embedded in celloidin or paraffin.

Sections stained in this way exhibit the following characteristics:

erythrocytes	-	pale green-blue
capillaries	-	red to magenta
muscle fibers	-	light blue to pale purple
collagen	-	purple
nerve	-	very pale purple
nuclei	-	bluish-purple (barely visible).

RESULTS AND DISCUSSION

We have used this staining procedure on sections of muscle which had been fixed in 10% neutral, buffered formalin and embedded in paraffin. These sections were cut, at a thickness of 15 μm, from blocks of tissue taken from several different muscles of cat, dog, guinea-pig, rat and rabbit. Most of these muscles had been perfused in vivo with an opaque, silicone elastomer (Microfil: Canton Biomedical Products, Inc.).

A typical section, taken from gastrocnemius of cat, is shown in Figure VI-1; the various components of this tissue exhibit the coloration described above. The capillaries are clearly visible, whether empty or containing either erythrocytes or Microfil. In our experience, sections stained by means of the procedure we have described, exhibit a very even staining throughout.

This method has been adapted from that presented in the 'Manual of Histologic and Special Technics' of the U.S. Armed Forces Institute of Pathology (1960). Two of the modifications were based on Culling (1963) and have been pointed out under Methods. Four major changes have been made which are worthy of comment:

1. The PAS reaction was carried out first, followed by the Luxol Fast Blue staining procedure. When the reverse procedure was employed the Luxol Fast Blue was washed out.
2. The use of haematoxylin staining has been avoided (Plyley & Groom, 1975).

3. The reducing solution, usually employed between the periodic acid and the Schiff's reagent steps, has been omitted (Culling pp. 228-29).
4. Leaving the tissue overnight in Luxol Fast Blue solution at 50°C was found to be unsuitable for skeletal muscle, leading to tissue disintegration. In fact, we found this step to be totally unnecessary.

We described previously (Plyley & Groom, 1975) a method for perfusion of the vascular bed with Microfil to identify the capillaries, and noted that incomplete filling often occurred in spite of the use of vasodilator drugs, high infusion pressure, and a substantial venous back-pressure. Identification of unfilled capillaries was made by the use of a modified Gomori trichrome method to stain bright red the erythrocytes they contained. However, the assumption was that few vessels would be devoid of both Microfil and erythrocytes. More recent studies have led us to believe that such is not the case and, in fact, the number of apparently 'empty' capillaries (whether by loss of contents during sectioning, or because they were sectioned through a plasma-gap between adjacent erythrocytes) may constitute a significant fraction of the total number. The use of Microfil injections at physiological pressure, coupled with serial sections stained by the PAS-Luxol Fast Blue and the modified Gomori trichrome procedures respectively, should now make it possible to determine the fraction of vessels in which blood was actually flowing at the time of injection.

Figure A-VI-1 - Transverse section, 15 µm thickness, of cat gastrocnemius muscle stained with the modified PAS-Luxol Fast Blue staining technique.

Legend:

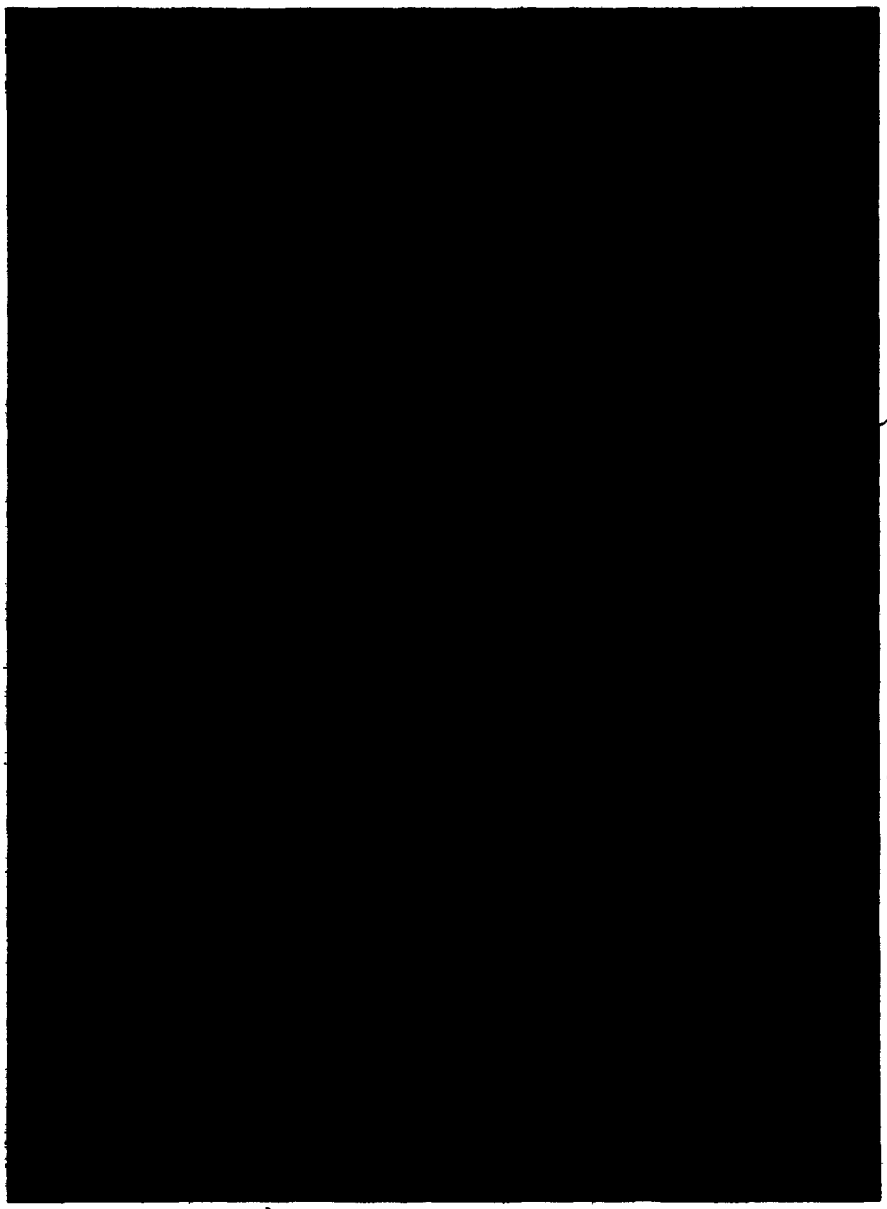
mf - muscle fibre

mc - capillary filled with Microfil

rc - capillary containing a red cell

ec - empty capillary

Scale: 1 cm ≡ 13 µm



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